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PATENT
Attorney Docket No. 9960.0003-00

**BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

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| In re Application of: | ) | |
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| J. Clark & C. Denning | ) | Group Art Unit: 1632 |
| | ) | |
| Application No.: 09/593,316 | ) | Examiner: Quan J. Ki, Ph.D. |
| | ) | |
| Filed: June 13, 2000 | ) | |
| | ) | |
| For: ANIMAL TISSUE FOR XENOTRANSPLANTATION | ) | Confirmation No.: 5627 |

**Mail Stop Appeal Brief--Patents**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

**TRANSMITTAL OF AMENDED APPEAL BRIEF (37 C.F.R. 41.37)**

Transmitted herewith is an Amended Appeal Brief (34 pages and Attachments 1-27) in this application in response to the Notice of Non-Complaint Appeal Brief issued by the Office on April 19, 2006.

Please associate these papers with this application.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: May 9, 2006

By: _____

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**BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

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| Filing Date: June 13, 2000 | Examiner: Quan J. Li, Ph.D. |
| Serial No: 09/593,316 | Confirmation No: 5627 |
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**AMENDED APPEAL BRIEF**

**Attention: Mail Stop Appeal Brief-Patents**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir,

Appellants file this Amended Appeal Brief in response to the Notification of Non-Compliant Appeal Brief dated April 19, 2006. The Notification indicated that appellants' brief was non-compliant because the brief does not contain copies of the decisions rendered by a court or the Board in the proceeding identified in the Related Appeals and Interferences section of the brief as an appendix thereto (37 C.F.R. 41.37(c)(1)(ix)).

Appellants enclose a Related Proceedings Appendix to Appeal Brief Under Rule 41.37(c)(1)(ix)). Appellants submit that this Amended Appeal Brief complies with 37 C.F.R. §41.37(c)(1)(ix).

Appellants hereby appeal from the final Office Action Mailed December 16, 2004, rejecting all claims under examination in this application. This paper constitutes appellants' Appeal Brief, as required under 37 CFR § 41.37.

## I. REAL PARTY IN INTEREST

The real party in interest for the claimed invention is Geron Corporation, a Delaware corporation, to which the application and the claimed invention has been assigned in their entirety.

## II. RELATED APPEALS AND INTERFERENCES

No other appeals or interferences are known by appellants or their representative that would directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

## III. STATUS OF CLAIMS

| | | |
|---|---|---|
| Claims 1-6: | Under examination: | Rejected under § 101 and § 112 ¶ 1 |
| Claim 7: | Withdrawn | |
| Claims 8-12: | Cancelled | |
| Claims 13-16: | Under examination: | Rejected under § 112 ¶ 1 |
| Claim 17: | Withdrawn | |
| Claims 18-21: | Cancelled | |
| Claim 22: | Withdrawn | |
| Claims 23-26: | Cancelled | |
| Claims 27-32: | Withdrawn | |
| Claims 33-37: | Under examination: | Rejected under § 101 and § 112 ¶ 1 |

## IV. STATUS OF AMENDMENTS

All amendments to the claims have been entered. No amendments were filed after the last final Office Action.

## V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention provides ovine cells, tissues, and animals that have been engineered for reduction or elimination of the carbohydrate epitope Galα(1,3)Gal, which ovine cells normally express. This epitope is important because it is a foreign antigen against which humans have naturally occurring antibody. Upon transplant of tissue bearing this epitope into an animal with naturally occurring antibody causes immediate hyperacute rejection. The idea is that elimination of Galα(1,3)Gal from the tissue will help render it more suitable for use in human transplantation therapy.

The epitope is produced by the enzyme α(1,3)galactosyltransferase (EC 2.4.1.124; abbreviated herein as "α1,3GT"), which adds galactose at the α(1,3) position to membrane-anchored Galβ(1,4)GlcNAc acceptor substance. Inactivation of the α(1,3)galactosyltransferase locus at both of the two alleles (a homozygous knockout) eliminates the enzyme from the cell, which in turn prevents the Galα(1,3)Gal epitope from being formed.

The invention is made possible by the discovery and isolation of the sheep α(1,3)galactosyltransferase (α1,3GT) gene and its genomic clone. The sequence of the gene is provided in the application (Example 1), and clones are deposited with the NCIMB in the United Kingdom in support of this application (page 51).

The specification illustrates how the α1,3GT sequences can be used to create targeting vectors (Example 3), which can then be used to inactivate the α1,3GT gene in isolated sheep fibroblasts from different strains (Examples 4 and 5). Heterozygous (single knockout) animals had been created from the targeted cells by the time the application was filed (Example 6), according to standard techniques in the field of animal cloning. Homozygous knockout animals can be made by using a double knockout cell in the cloning process, or by cross-breeding heterozygous knockout animals (pages 37-41). The cells and tissue made during the course of this work are then characterized (pages 41-43) to verify that they have the characteristics of the claimed invention — either at the genetic level (inactivation of the α1,3GT gene) or at the phenotypic level (reduced expression of the Galα(1,3)Gal epitope).

## VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 1-6, 13-16, and 33-37 stand rejected under the enablement requirement of 35 USC § 112 ¶ 1. The basis of this rejection is essentially that the specification fails to provide a working example of a homozygous α1,3GT knockout adult sheep (even though some of the claims refer only to isolated tissues or cells, such as could be created in culture).

The specification provides the sheep α1,3GT gene sequence for the first time, which enables the reader to make α1,3GT knockout cells, tissues, and animals according to standard methods used in other species. The standard methods needed for the reader to carry out the full scope of the claimed invention are referenced in the specification. Nevertheless, the Examiner has maintained that the specification is not enabling without a working example because the relevant art is too unpredictable.

Claim 16 also stands rejected under the enablement requirement of § 112 ¶ 1 on the assertion that the α1,3GT knockout tissue of the invention does not solve all the problem of xenotransplantation.

Claims 1-6 and 33-37 stand rejected under 35 USC § 101 as having no credible utility. The basis of this rejection is essentially that the claimed invention is not enabled, and so cannot have any utility.

History of the application

The claims under examination were initially rejected for not having a working example under the written description requirement of § 112 ¶ 1. Appellants appealed on June 4, 2003. The application was pulled back into regular prosecution on January 30, 2004, so that the Examiner could make essentially the same rejection under the enablement requirement of § 112 ¶ 1, and the utility requirement of § 101. The written description rejection has been withdrawn.

## VII. ARGUMENT

This application provides the sequence for the sheep α(1,3)galactosyltransferase ("α1,3GT") gene, which was cloned and characterized for the purpose of making α1,3GT sheep. α1,3GT knockout mice had already been produced in other labs, and efforts to make α1,3GT pigs were already under way. Using genetically modified stem cells (the technique used to make the mice) is not amenable to making knockout sheep — but nuclear transfer cloning in sheep was a well established technology at the time of filing, and has been used since by several labs to make α1,3GT knockout pigs.

The sheep sequence is the latchkey that enables knockout sheep to be made in the same fashion as other animals. This is the kernel of the inventive aspects of this disclosure, and places into the hands of the public the entire scope of the claimed invention by using standard technology.

The Examiner has taken the position that none of the claims of this invention can be enabled without a working example of a homozygous α1,3GT knockout adult sheep (even though some of the claims refer only to isolated tissues or cells, such as could be created in culture). In support of this position, the Examiner has selectively interpreted published academic articles to indicate that animal cloning is a difficult and unpredictable field.

Appellants do not deny that cloning is both time-consuming and expensive. However, neither of these aspects imply that completion of the project would require undue experimentation defined by *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). In fact, Geron decided to stop funding of this project part way through because of the costs involved. Preliminary results of the project (cloned α1,3GT knockout fetuses) were then published in an academic journal (Denning et al., Nat. Biotechnol 19:559, 2001) — not as evidence of failure, but to demonstrate the progress made. Appellants have no doubt that the making of α1,3GT knockout sheep can be completed as described in the application as a matter of routine experimentation when the project is resumed. Naturally, the delay in obtaining a patent for this invention is not helpful in obtaining the financial support of another backer.

It is appellants' position that rejection of the claimed invention under 35 USC §§ 101 and 112 ¶ 1 for lack of working examples of all embodiments of the invention is improper.

An instructive parallel can be drawn from the patentability requirement for claims covering human treatment, and the manufacture of pharmaceutical products for human use. The courts and the Office have long recognized that an applicant does not need to have a working example of the treatment in a human subject in order for the invention to be patentable.

> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of patent laws. *Scott [v. Finney]*, 32 USPQ2d 1115. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily induces the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer. *In re Brana,* 34 USPQ2d 1436 (Fed. Cir. 1995).

Similarly, appellants for the invention claimed here should not be denied patent coverage, just because a homozygous knockout sheep was not obtained before the application was filed. Again, were the patent office require a full working example of the entire scope of this type of invention in order to prove utility and enablement, the associated costs would eliminate an important incentive to create products for use in regenerative medicine and tissue transplantation.

Appellants should not be penalized for disclosing their invention before they completed reduction to practice of all the embodiments. To the contrary: an objective of the patent law is to disseminate advances in the art and thereby provide a public benefit as soon as possible. The inventors of the claimed invention fully entered into the spirit of the public policy objective by filing their patent disclosure soon after critical elements of the invention had been made (isolation and characterization of the sheep α1,3GT gene). They knew full well that this places the invention in the hands of the public through the implementation of standard cloning technology.

For these reasons, appellants respectfully submit that the claimed invention meets all the patentability requirements of 35 USC §§ 101 and 112.

The following remarks address in more detail the arguments made during prosecution.

Rejections under 35 USC § 112 ¶ 1

Claims 1-6, 13-16 and 33-37 stand rejected under the enablement requirement of § 112 ¶ . The central issue appears to be the fact that the specification does not indicate that a homozygous α1,3GT knockout sheep actually having been made.

Of course, the Board will recognize that there is no legal requirement that an actual working example be provided in the specification in order for a patent disclosure to be enabling.

> It is well established in the law that a specification can adequately describe the manner and process of making an embodiment of an invention, whether or not it has actually been conducted. Use of prophetic examples does not make a patent non-enabling. The burden is on the person challenging the patent to show . . . that the prophetic examples together with other parts of the specification are not enabling. *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 224 USPQ 409 (Fed. Cir. 1984).
>
> Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided though broad terminology or illustrative examples. *In re Wright,* 27 USPQ2d 1510 (Fed. Cir. 1993).

It is appellants' position that the claims are fully described and enabled, *inter alia* because the only elements missing from the working examples can be achieved as a matter of routine experimentation by the skilled reader.

Specifically, the description provides the newly discovered nucleotide sequence of the sheep α1,3GT cDNA (SEQ. ID NO:1) and the α1,3GT gene (SEQ. ID NOs:14 to 25). This is supported by a biological deposit (NCIMB Accession No. 41056) comprising cloned genomic DNA. Example 3 illustrates how the α1,3GT sequences can be used to create targeting vectors, which can then be used to inactivate the α1,3GT gene in isolated sheep fibroblasts from different strains (Examples 4 and 5). Heterozygous (single knockout) fetal sheep were created from the targeted cells by the time the application was filed (Example 6), according to standard techniques in the field of animal cloning.

Homozygous knockout animals can be made by using a double knockout cell in the cloning process, or by cross-breeding heterozygous knockout animals (pages 37-41). The cells and tissue made during the course of this work are then characterized (pages 41-43) to verify that they have the characteristics of the claimed invention — either at the genetic level (knockout of the α1,3GT gene) or at the phenotypic level (reduced expression of the Galα(1,3)Gal epitope). Furthermore, the homozygous knockout cells of claims 4, 5, and 33-37 can be made without making a homozygous knockout animal, or doing any animal cloning at all (page 40, line 20 to page 41, line 13).

In requiring appellants to provide a working example of a homozygous α1,3GT knockout sheep, the Examiner has relied on the assertion that the area of making knockout animals is generally unpredictable. However, the Examiner has raised no specific technical issue why the making of a knockout sheep should pose special technical difficulties — difficulties that were not experienced in making α1,3GT knockouts in two other animal species.

Accordingly, the Office has not met its burden of establishing a *prima facie* case for non-patentability under 35 USC § 112 ¶ 1.

Nevertheless, in responding to the Office Actions, appellants have explained how it is possible to make a homozygous α1,3GT knockout sheep without undue experimentation, and why the phenotype is predictable.

***1. Sheep cells having an inactivated α1,3GT allele can readily be produced.***

One embodiment of the invention disclosed in this application are sheep cells in which the α1,3GT gene has been inactivated. This is claimed directly in claim 4 and claims 33-37. As explained in the specification, α1,3GT knockout cells can also serve as nuclear donors for the making of knockout animals by nuclear transfer (animal cloning).

The application newly provides the sheep α1,3GT gene, and describes and illustrates how to make targeting constructs for the purposes of creating α1,3GT knockout cells. Methods for using gene sequences to inactivate the corresponding gene in living cells are described extensively in the art.

Figures 9-15 in the application provide illustrations of targeting constructs based on the α1,3GT sequence that will inactivate the α1,3GT gene by removing exon sequences. Figure 16 illustrates the successful targeting and deletion of Exon 4, using the p0054 vector[1]. In Example 5, similar targeting was demonstrated using the same constructs on a different sheep strain cell line[2].

Thus, the skilled reader may use the constructs provided in the working examples, or design their own knockout strategy, by applying the sheep α1,3GT sequence using standard procedures in order to make the knockout cells of the invention.

***2. Sheep cells that are homozygously inactivated at the α1,3GT locus can readily be produced***

Inactivating a single α1,3GT gene in a cell creates a heterozygous knockout. In order to prevent expression of the Galα(1,3)Gal epitope on the cell surface, both α1,3GT alleles must be inactivated (a homozygous knockout). As described in the specification, homozygous α1,3GT knockout cells according to claims 4 and 33-37 can be made using cultured cells, or by harvesting cells from a homozygous knockout animal.

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[1] The Office Action of May 21, 2002 (page 7) indicates concern that the specification provides no direct evidence for successful targeting of Exons 8 and 9. Nevertheless, targeting Exons 8 and 9 should be achievable without undue experimentation. In any event, it is not necessary to target Exons 8 and 9 to practice the claimed invention. It is only necessary to eliminate the translation start or remove enough of the gene to prevent the gene product from being functional. The targeting of Exon 4 (as effected in the working examples), or some other portion of the gene, or various portions in combination will be sufficient for the purpose of inactivating the α1,3GT gene in a sheep cell. The enablement requirement is met if the description enables *any mode* of making and using the claimed invention. *Engel Industries, Inc. v. Lockformer Co.*, 20 USPQ2d 1300 (Fed. Cir. 1991), emphasis added.

[2] The Office Action of November 23, 2001 (page 8) says that the specification is enabled for homozygous inactivation of the α1,3GT gene in the Finn Dorset strain of sheep, but not in other strains. It is recognized in the art of homologous recombination that a mismatch of about 1% (i.e., identity of about 99%) is well tolerated when using targeting constructs to cause gene inactivation. The difference between strains of the same mammalian species typically falls within this range. The α1,3GT sequence and targeting constructs used in the working examples were obtained from Black Welsh Mountain fibroblasts (Example 1). They have been used to successfully inactivate the α1,3GT gene in both Black Welsh Mountain sheep cells (Example 4), and Finn Dorset sheep cells (Example 5). This confirms that the claimed invention is enabled for different strains within the ovine species.

An extensive description of how to make α1,3GT homozygous knockout cells in culture is provided in the specification beginning on page 40, line 20. Several techniques are explained, including sequential targeting of the two alleles by any one of these methods:

- Using a step-wise increase in antibiotic concentration to knock out both alleles (page 40, lines 24-27, citing U.S. Patent 5,589,369)
- Using two different antibiotics to sequentially knockout each allele (page 40, line 27 to page 41, line 4, referring to targeting constructs shown in Figures 9 and 11)
- Knocking out the first allele, and then retargeting and selecting homozygous knockout cells using an antibody that recognizes the Galα(1,3)Gal epitope (page 41, lines 8-13)

The claims have been rejected under 35 USC § 112 ¶ 1 on the assertion that a α1,3GT knockout sheep cannot be made. Recent Office Actions refer back to publications directed towards animal cloning to support the contention that homozygous α1,3GT knockout cells are difficult to make in culture[3]. On this basis, the Examiner indicates that the claims to knockout cells are not enabled[4]. Again, the Examiner is essentially requiring appellants to provide evidence of actual reduction to practice in order to meet the requirements of § 112 ¶ 1.

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[3] The Advisory Action of May 6, 2003, and subsequent Office Actions refer to the article by Phelps et al., Science 299, 411-414, 2003. Heterozygous α1,3GT knockout pig cells were targeted a second time to obtain homozygous knockout cells, for the purpose of making homozygous knockout pigs. As it turns out, the second allele was inactivated not by homologous recombination, but by a fortuitous mutation event. Of course, this doesn't mean that homologous recombination doesn't' work; only that the single positive event ultimately cloned out of the system happened to have been achieved in an unexpected manner. The Advisory Action quotes col. 3 of page 413, which attributes the finding of the cell with the desired phenotype to a "powerful selection method". The method is described in col. 1 of page 412, and involves selecting targeted cells using a toxin that eliminates cells still expressing the Galα(1,3)Gal epitope. *An equivalent method is described in the specification on page 41, lines 9-11,* which teaches using an epitope-specific antibody to cause complement-mediated lysis of cells still expressing the Galα(1,3)Gal epitope. Thus, the patent application provides all the tools needed for the skilled reader to make a α1,3GT knockout cells in a manner that is equivalent to the Phelps method. As explained in Section 4 below, the Phelps article also confirms that this patent application enables the making of a α1,3GT knockout sheep.

[4] This is contrary to the position taken in the Office Action of November 23, 2001. On page 8, the Office Action says that the specification is enabled for homozygous inactivation of the α1,3GT gene in Finn Dorset sheep.

U.S. Patent 5,589,369 referred to in the specification describes and claims a system for making homozygous knockout cells[5]. Since this is an issued patent, 35 USC § 282 requires that the patent be presumed valid, and therefore enabled under 35 USC § 112 ¶ 1. The Examiner has not explained why the making of α1,3GT knockout cells would pose special difficulties that prevent the method of U.S. Patent 5,589,369 (or the other alternatives described in the specification) from being implemented as a matter of routine experimentation.

The steps referred to in Sections 1 and 2 are sufficient to enable the full scope of the cells in claims 4 and 33-37.

***3. Animals having an inactivated α1,3GT allele can readily be made from α1,3GT inactivated donor cells by nuclear transfer***

Some claims in the application involve or are facilitated by the making of a cloned animal from α1,3GT knockout cells. A central aspect of this invention relates to the discovery and characterization of the sheep α1,3GT gene, and its use for inactivating the α1,3GT gene inside cells. The α1,3GT knockout cells explained in Sections 1 and 2 above can be made into knockout animals using standard methods known in the art.

Several methods are available for making genetically modified animals from genetically altered cells. The specification explains extensively on pages 37-41 that animals can be cloned from a suitable donor cell by nuclear transfer. This is proven technology that created Dolly the sheep. The nuclear transfer method has been fully described and enabled in issued U.S. patents 6,147,276 and 6,252,133 (Campbell & Wilmut, Roslin Institute).

There is no reason to believe that genetically altering the donor cell would affect its suitability as a nuclear donor. To the contrary. A number of published experiments confirm

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[5] US 5,589,369 (Seidman and Jakobovits, Cell Genesys) is entitled "Cells homozygous for disrupted target loci". Claim 1 covers "A method for making diploid mammalian cells homozygous for disrupted target loci . . . comprising . . . (a) introducing into diploid mammalian cells a construct . . . comprising a selectable marker gene . . . (b) growing the cells . . . in said selective medium at a first level of selective agent; (c) subjecting the population of cells . . . to a level of selective agent greater that said first level . . . and (d) isolating said cells . . ."

that cloned animals may readily be made from genetically altered cells according to the Campbell & Wilmut method.

- Uchida et al. (Transgenic Research 10:577, 2001) report the production of transgenic miniature pigs by pronuclear microinjection. The Huntington gene cloned from miniature pig, was linked to rat enolase promoter, and injected into pronucleus of fertilized eggs. Several of the offspring were determined to have the transgene by PCR and Southern analysis.
- Bondoli et al. (Molec. Repro. Dev. 60:189, 2001) report cloned pigs generated from cultured skin fibroblasts derived from a boar with an H-transferase transgene. Two healthy piglets resulted from nuclear transfer by fusion of fibroblasts that had been extensively cultured with enucleated oocytes.
- Lai et al. (Molec. Repro. Dev. 62:300, 2002) report a transgenic pig expressing green fluorescence protein. Fetal-derived fibroblast cells were transduced with the GFP gene, and then cloned into porcine oocytes. A healthy transgenic piglet was obtained that expressed GFP.
- McCreath et al. (Nature 405:1004, 2000) report transgenic sheep made by nuclear transfer from fibroblast donors in which different transgenes were targeted into the alpha1(I) procollagen locus.
- Lai et al. (Science 295:1089, 2002) report production of α(1,3)galactosyltransferase knockout pigs by nuclear transfer cloning. The pigs were produced by nuclear transfer, using clonal fetal fibroblast cell lines as nuclear donors.
- Dai et al. (Nature Biotech 20:251, 2002) also report production of α(1,3)galactosyltransferase knockout pigs by nuclear transfer cloning. The pig α1,3GT gene was disrupted in both male and female porcine primary fetal fibroblasts, which were then used for nuclear transfer. Six clonal fetal piglets were obtained, of which five were normal weight and apparently healthy. Southern blot analysis confirmed that the five piglets contained one disrupted α1,3GT allele.

- Denning et al. (Nat. Biotechnol 19:559, 2001) describe the deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Eight pregnancies were maintained to term and four PrP-/+ lambs were born.
- Schnieke et al. (Science 278:2130, 1997) report production of human factor IX transgenic sheep. Ovine fibroblasts were transfected with the human factor IX gene, and used as donors for nuclear transfer to enucleated oocytes. Six live transgenic lambs were born, of which three contained the factor IX gene.
- Cibelli et al. (Science 280:1256, 1998) transfected bovine fibroblasts with a marker gene, which were then fused to enucleated mature oocytes. Out of 28 embryos transferred, three health transgenic calves were isolated.
- Kuroiwa et al. (Nature Genetics 36:775, 2004) have produced cattle that are homozygous for inactivation of the bovine gene encoding IgM μ-chain (IGHM). Cells were targeted on one allele and used as donors to make heterozygous fetuses. Tissue was harvested, retargeted using *non-isogenic* vectors, and used to make homozygous knockout animals. *Five rounds* of harvesting fetal tissue, genetic modification, and nuclear transfer, produced tissue with this genotype: homozygous inactivation of IGHM, containing a *Cre* transgene, and homozygous inaction of the PRNP gene (responsible for mad cow disease). Nine pregnancies having the five modifications have survived beyond 60 days. Kirin Pharmaceuticals intends to use these animals for producing human IgM antibody for therapy.
- Ramsoondar et al. (Biol. Reprod. 69:437, 2003) reported the production of pigs containing both a α1,3GT knockout and an α(1,2)fucosyltransferase transgene. Donor fibroblasts *already contained a genetic modification* — the α1,2FT transgene. They were targeted at the α1,3GT locus with *non-isogenic* DNA, producing cells that had two genetic modifications — which were then used successfully for nuclear transfer.

- Sendai et al. (Transplantation 76:900, 2003) reported production of heterozygous α1,3GT cattle. One fetus was produced from 24 cloned embryos. A fibroblast cell line was established from the fetus for second round targeting, intended for cloning into a homozygous knockout.

These references confirm that pigs, sheep, and cattle can all be cloned by the Campbell & Wilmut method using genetically altered cells to make genetically modified animals. References 5, 6, 7, 11, and 12 are of particular interest, because they illustrate that heterozygous α1,3GT knockout animals can readily be made by nuclear transfer of heterozygous knockout cells. All the evidence of record indicates that the cloning of sheep according to the Campbell & Wilmut method is no more difficult if the cell used as the nuclear donor has been genetically altered.

***4. Animals that are homozygous for inactivated α1,3GT can readily be produced***

The skilled reader has at least three options by which to make a sheep in which both α1,3GT alleles have been inactivated:

1. Homozygous knockout cells can be made in culture as explained in Section 2 above. They are then used as nuclear donors to make homozygous knockout animals by the animal cloning method of Campbell & Wilmut (specification: page 37-41).
2. As an alternative, a nuclear donor cell with α1,3GT inactivated on one allele is used to produce a heterozygous knockout animal. Cells are harvested for a second round of targeting. This generates homozygous knockout cells, which can then be used to generate homozygous knockout animals by a second cloning event (specification: page 41, line 17). The article by Kuroiwa et al., *supra,* provides an illustration of genetically modified cattle made by five rounds of sequential cloning.

3. Another alternative again involves making a heterozygous knockout animal to start. However, in this case, heterozygous knockout animals are simply cross-bred to produce a homozygous knockout animal (specification: page 41, line 14). This requires time for breeding the second generation, but in some ways is the most straight-forward option.

Since these technologies are all in wide-spread general use, the only relevant question in relation to the invention claimed in this application is *whether knocking out both α1,3GT alleles would somehow compromise the viability of the animal.*

In fact, we know this not to be the case. Humans and other Catarrhine primates are exceptions amongst mammalian species as not having an expressed α1,3GT gene. We seem to get along quite well without it. The α1,3GT gene has been obtained from two other species that normally express it, and used to create homozygous knockouts without difficulty.

Furthermore, the α1,3GT gene has successfully been knocked out in at least two other mammalian species that normally express it.

- U.S. Patent 5,849,991 (Cols. 48-57) describes the isolation of the mouse α1,3GT gene, and then using it to make homozygous α1,3GT knockout mice.
- Phelps et al., Science 299, 411-414, 2003 describe production of homozygous α1,3GT knockout pigs using the pig α1,3GT gene[6].
- Kolber-Simonds et al. at Immerge BioTherapeutics (Proc. Natl. Acad. Sci. USA 101:7335, 2004) are *another group* to report production of *homozygous α1,3GT knockout pigs.*

The α1,3GT knockout mice of the '991 patent were made by targeting one α1,3GT locus in mouse embryos to make heterozygous knockout, and then cross-breeding to obtaining the homozygous knockouts (option 3, above). Such mice have been used extensively in labs

[6] The pig α1,3GT gene sequence had already been disclosed in U.S. Patent 5,821,117. This patent includes claims both to the gene sequence *and to cells having an inactivated α1,3GT gene.*

around the world for immunological and transplant studies, and have the usual features of animals of the murine species — with the exception that they lack the Galα(1,3)Gal epitope on their cells.

The knockout pigs of Phelps et al. were also made according to the methods described in this patent application (option 2). First, the pig α1,3GT gene was used to make heterozygous knockout donor cells, which were then used to clone heterozygous knockout pig (page 412, col. 1; described in the present application *inter alia* on page 38, line 5 to page 40, line 19; and page 41, line 22 to page 42, line 5). Next, homozygous knockout cells were made by a targeting the other allele in the donor cells using a knockout vector, and selecting cells deficient in the Galα(1,3)Gal surface antigen (page 412, col. 1; described in the present application *inter alia* on page 41, lines 9-13 and 17-20; and page 42, lines 6-16). Finally, double knockout cells were used as donor cells for nuclear transfer to produce homozygous knockout animals (abstract; described in the present application *inter alia* on page 38, line 9 to page 40, line 19).

Four double-targeted female piglets were produced by Phelps et al., of which three had α1,3GT inactivated on both alleles (page 412, col. 3 ff).

Similarly, the knockout pigs of Kolber-Simonds et al. were made by knocking out the two α1,3GT alleles in two sequential rounds of cloning. Cell lines established from heterozygous knockout cells were selected for spontaneous inactivation of the second allele using antibody staining. They were then used successfully as nuclear donors: 48 transfers resulted in 17 pregnancies, and 4 homozygous α1,3GT knockout piglets.

Based on the precedents of humans, other Catarrhine primates, and homozygous knockout mice, and pigs, there is no reason to believe that homozygous knockout sheep would not be equally viable, and equally straight-forward to produce by any one of the three approaches described in the specification.

Nevertheless, in the Office Action dated January 30, 2004 states that *contrary to the pig, knockout of α1,3GT gene kills ovine fetuses* (page 17). This statement is entirely without foundation. The Galα(1,3)Gal antigen has no known biological function that is required for survival. All upper primates lack α1,3GT, and seem to get along quite well without it.

Homozygous α1,3GT knockouts have been made in the mouse and the pig without difficulty. There is no evidence of record to indicate that sheep are any different.

The notion that knocking out the α1,3GT gene will specifically kill sheep is not substantiated in any cited reference, and appears to come from the personal knowledge or speculation of the Examiner. For this reason, appellants made a formal request for an Examiner's Affidavit on August 2, 2004, pursuant to 37 CFR § 1.104(d)(2) and MPEP § 2144.03. This request was denied, but the same assertion was made in the Office Action dated December 24, 2004.

***5. Cells from homozygous knockout animals will have cells and tissues lacking the Galα(1,3)Gal xenoantigen***

As described in the specification, the α1,3GT gene is responsible for forming the Galα(1,3)Gal xenoantigen in non-Catarrhine mammals. An animal that is homozygous for inactivation of the α1,3GT gene would therefore lack the enzyme responsible for making the Galα(1,3)Gal epitope.

The homozygous knockout mice in U.S. Patent 5,849,991 confirm this expectation. Peripheral blood monocytes and splenocytes from the homozygous knockouts were analyzed for presence of the Galα(1,3)Gal antigen using the IB4 lectin, in a manner comparable to what is described in the specification of the present application on pages 41-43. Wild-type mice showed high degree of staining, while knockout mice showed minimal staining, confirming that the tissue was essentially devoid of the Galα(1,3)Gal antigen (U.S. 5,849,991, Cols. 48-52). As expected, since Galα(1,3)Gal is not a self-antigen in these mice, they form naturally occurring antibody against it, as do humans (Chong et al., Transpl Immunol 8:129-37, 2000).

The homozygous knockout pigs in the article by Phelps et al. This is shown in are devoid of antibody-detectable Galα(1,3)Gal. See Fig. 1., clones B1-1, B1-2, and B1-4 (the three correctly targeted clones), and Fig. 2.

Similarly, Dor et al. (Transplantation 78:15, 2004) showed that the five α1,3GT knockout pigs made in the manner of Kolber-Simonds et al. had essentially no expression of

Galα(1,3)Gal as determined by IB4 staining (Table 1, Figure 1) and have naturally occurring cytotoxic anti-Galα(1,3)Gal antibody (Figure 3).

Apparently the Examiner agrees that homozygous α1,3GT knockout sheep will lack the Galα(1,3)Gal antigen on their cells. The Office Action dated December 24, 2004 (page 17) states: "This is not an issue once a homozygous α1,3GT knockout animal is made."

***Predictability of the Art***

In requiring appellants to provide full reduction to practice of a homozygous α1,3GT knockout sheep, the Examiner asserts that the "physiological art" in general, and the art of making genetically altered animals in particular, is unpredictable. In support of this assertion, two publications have been put forward in the Office Actions.

The Office Action of November 23, 2001, refers to an article by Linder et al. (Lab. Anim. NY 30:34, 2001) as indicating that the resulting phenotype of a targeted gene mutation would vary among different strains of animals because of the collective effect of different genes in the host. The issue raised in the article is that inactivation of a gene may generate different phenotypes in particular inbred strains of mice. This is because the genes exemplified do not directly generate the phenotype being measured, but cause the phenotype to change by complex interaction with other gene products.

In contrast, the α1,3GT gene targeted in the present invention is directly responsible for generating the enzyme that builds the Galα(1,3)Gal epitope[7]. Accordingly, none of the concerns raised in the Linger article are relevant. The homozygous α1,3GT knockout mice described in U.S. Patent 5,849,991 were made by breading heterozygous knockout mice, and

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[7] Table 2 in the Linder article shows that knocking out the gene for IL-2 can cause splenomegaly, inflammatory bowel disease, or generalized autoimmune disease, depending on the genetic background. A similar observation is made for *ob/ob* obese mice, which have a homozygous mutation in the leptin gene. The target genes in these studies are both endocrine molecules (IL-2 and leptin) which mediate a complex response pathway between different cells. In contrast, the present invention is directed at inactivating a gene that puts a terminal sugar residue onto the carbohydrate substrate N-acetyl lactosamine, which all ovine animals express. Accordingly, no inter-strain variation is expected. Another issue raised in the Linder article is that a cross-over event that occurs during breeding may separate a mutant gene from the phenotype being used to follow the breeding, if the phenotype is not directly encoded by the mutated gene. This is not a concern for the present invention, because the presence of an inactivated target gene can be detected directly — either by PCR analysis (Figures 16 and 17), or by detection of the Galα(1,3)Gal epitope on the animal's cells.

the line has continued to bred true. All evidence indicates that α1,3GT knock-out animals reliably breed towards absence of the Galα(1,3)Gal epitope, as expected.

The Advisory Action of May 6, 2003, refers to the article by Denning, Clark, et al. (coinventors on this patent application) entitled *Deletion of the α(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in the sheep* (Nature Biotechnol. 19:559, 2001). The Examiner is apparently concerned that the first α1,3GT knockout sheep clones died *in utero*. But the Denning article was only a preliminary report. It does not support the contention that gene targeting in sheep is an uncertain process.

On the contrary — the article provides several illustrations of the viability of the claimed invention:

- Sheep cells can be correctly targeted for inactivation of the α1,3GT gene. See Figure 2, panel (A); and Table 1. This provides a direct illustration of the making of the knockout cells covered by claims 4 and 33-37.
- Targeted cells can be used for nuclear transfer. There are three examples: a) the α1,3GT knockout cell gave rise to viable embryos; b) the PRP knockout cells gave rise to 3 live births; c) viable animals have been produced that were successfully targeted at the COL1A1 locus (ref. 5, discussed on page 559, col. 1).
- *Knocking out the α1,3GT gene does not decrease viability of the embryo.* See the data in Table 2. Nuclear transfer with untransfected donor cells (7G65F4) gave rise to 5 viable fetuses at day 60 in 33 attempts (a 13% success rate). Nuclear transfer of cells treated with the α1,3GT vector but not inactivated (4H2) gave rise to 2 viable fetuses in 23 attempts (an 8% success rate). Nuclear transfer of cells containing an inactivated α1,3GT gene (3C6 and 5E1) gave rise to 5 viable fetuses in 21 attempts (a 19% success rate). Ergo, the success rate for cloning sheep by nuclear transfer is not further reduced by knocking out the α1,3GT gene. If anything, there was actually an improvement in cloning frequency using the correctly targeted cells.

Thus, the Denning article confirms that the generation of α1,3G knockout sheep *poses no undue difficulty* beyond what is usually entailed in producing cloned knockout mammals by nuclear transfer. The method has been used successfully for recombination at the COL1A1 locus in sheep (McCreath et al., Nature 405:1066, 2000), and the α1,3GT locus in pigs (Phelps et al., *supra*).

The sheep α1,3GT knockout project reported in the Denning article lost its funding, and it is for this reason that knockout sheep were not ultimately produced by these authors. There is no question that large animal cloning is a costly and time-consuming process, whether or not *the nuclear donor cell has any kind of genetic alteration.* But that does not mean that the claimed invention is in any way inadequately described or enabled. The cloning step required to complete the invention claimed in this application, while costly, is entirely straight forward. *It can be accomplished without undue experimentation,* well within the Wands standard[8]. There is nothing missing from the specification that the skilled reader needs in order to put this invention into practice[9].

***§ 1.132 Declaration by Dr. Ian Wilmut***

A Declaration by Ian Wilmut, Ph.D., O.B.E., F.R.S., was filed in this application under 37 CFR § 1.132 on September 23, 2004. A copy of the Declaration accompanies this Appeal Brief. It provides further support for appellants' position that the claimed invention is enabled by the application as filed.

Dr. Wilmut explains that genetically modified animals can readily be made by the methods described in U.S. patents describing nuclear transfer, and provides a number of illustrations. He also explains that the early death of α(1,3)galactosyltransferase knockout

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[8] *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). In *Wands,* the patent application claimed monoclonal antibodies of a particular specificity and affinity. The PTO contended that only 2.8% of the hybridomas obtained were proven to fall within the claim, and thus the claim was not enabled. The Court held that Wands was fully enabled, because it was standard practice to screen negative hybridomas in order to find one that makes the desired antibody.

[9] Except, of course, funding. But 35 USC § 112 ¶ 1 only requires the applicant to provide the skilled reader with the knowledge required to make and use the invention — not the financial resources that may be needed to complete the project.

sheep fetuses reported in the Denning article is often observed in cloned animals, and not attributable to an effect from the α1,3GT gene. He explains that the sheep α1,3GT gene provided in this application can be used to make α1,3GT knockout sheep, just as the pig α1,3GT gene has been used to make α1,3GT knockout pigs.

Thus, even if it takes several attempts to obtain a working example because of a low frequency of successful cloning events, there is no undue experimentation involved. The skilled reader simply repeats the procedure until a α1,3GT knockout sheep is obtained.

***Patentability of individual claims***

As already explained, techniques suitable for preparing α1,3GT knockout animals are generally known in the art, and referenced in the specification. It has not previously been possible to make α1,3GT knockout sheep, simply because the sheep α1,3GT gene was not previously available. Now that the sheep α1,3GT gene has been discovered and characterized, it is straight forward to produce sheep tissue which is devoid of Galα(1,3)Gal, or which has been inactivated for the α1,3GT gene on one or both alleles, using techniques already proven to be effective in the mouse and the pig.

Claims 6, 14, and 15 cover an ovine animal that is homozygous for inactivation of the α1,3GT gene. They meet the enablement requirements of 35 USC § 112 ¶ 1 for reasons already explained. Briefly, the specification provides the sheep α1,3GT gene, α1,3GT targeting constructs, and α1,3GT knockout sheep cells. α1,3GT knockout sheep can be made by applying standard animal production technology referred to in the specification.

Claims 4 has different requirements under 35 USC § 112 ¶ 1. Heterozygous and homozygous cells can be made in culture without producing a knockout animal.

Claims 33-37 specify that the cell of claim 4 is a fibroblast, kidney cell, hepatocyte (liver cell), cardiac cell, or islet cell. Fibroblasts having the required property, made by homologous recombination of cultured cells, are shown in the specification as working Examples 4 and 5 (page 46 ff.). The other cell types can be produced by homologous recombination using the α1,3GT targeting construct with cultured cells of the particular tissue type, in the same fashion.

Claim 5 depends from claim 4, and requires only that the process for making the cell involve at least one nuclear transfer event. This may involve the making of a cloned animal, or just the making of a single cell or cell culture by nuclear transfer.

Claim 13 depends from claim 4, and covers the use of heterozygous or homozygous α1,3GT knockout cells for making homozygous α1,3GT knockout sheep.

Claim 3 covers a cell or tissue that does not express α1,3GT. Claims 1 and 2 cover tissue devoid of the Galα(1,3)Gal epitope. Again, cells having these properties can be harvested from a α1,3GT knockout animal, or produced *in vitro* by genetic manipulation of cultured cells.

Claim 16 depends from claim 1, calling out a particular use of the cells of claim 1 for transplanting into a mammal having circulating antibody against Galα(1,3)Gal determinants — e.g., a non-cattharine primate, or a α1,3GT knockout mouse or pig.

***Summary***

The Office has not established a prima facie case for lack of enablement for the claimed invention. Heterozygous knockout cells and sheep have been made using the α1,3GT targeting vectors described in the specification. There is no basis to believe that making a homozygous knockout will compromise the viability of the animal in any way. In fact, there is abundant evidence from other species that the making α1,3GT knockouts is straight forward.

Appellants should not be required to provide complete actual reduction to practice in order to demonstrate enablement of the invention, since this is not the legal standard. Methods needed to practice the full scope of the claimed invention are known in the art, and can be implemented by the skilled reader without undue experimentation. The claimed invention is placed into the hands of the public because the critical component needed to make this work in sheep — namely the sheep α1,3GT gene — is provided in the disclosure for the first time.

Thus, the claimed invention is fully described and enabled in the specification, thereby complying with the patentability requirements of 35 USC § 112 ¶ 1.

Further rejection of Claim 16 under § 112 ¶ 1

Claim 16 also stands rejected under 35 USC § 112 ¶ 1 as not being enabled by the specification, because even if ovine tissue devoid of Galα(1,3)Gal determinants could be produced, it would not solve all of the issues that trigger a xenograft response.

Appellants disagree. The application solves the problem of the Galα(1,3)Gal present on sheep tissue, which would generate hyperacute rejection upon transplantation to a human. The skilled reader would recognize that other issues in transplantation therapy should also be addressed, for example, by the use of immunosuppression and other supportive care that are standard in the transplantation setting. Standard immunosuppressive drugs such as cyclosporin A have a long established track record for overcoming immune rejection of grafted organs, and would be adopted as a matter of course in the use of the claimed invention.

In fact, xenografting has been an established protocol for cardiac valve replacement for almost 40 years. Long-term postoperative survival rates have been between 78% and 94%, depending on what procedure is performed (Stinson et al., J. Thorac. Cardiovasc. Surg. 73:54, 1977; Angell et al., Ann. Thorac. Surg. 28:537, 1979). The success is not impaired by the fact that the value tissue used is taken from animals that normally express Galα(1,3)Gal. The Office Action does not explain why the tissue of this invention would not be effective for therapeutic applications such as cardiac valve therapy — regardless of the epitopes expressed.

Furthermore, Costa et al. (FASEB J. 17:109, 2003) reported experiments in which xenograft survival was tested in a model where cartilage was transplanted from transgenic pigs to α1,3GT knockout mice. The pig tissue expressed the transgene α(1,2)fucosyltransferase which reduces but does not eliminate expression of Galα(1,3)Gal. Control pig cartilage grafted into the mice was rejected in several weeks in a cell mediated response. In contrast, the mice receiving the tissue with the transgene showed a markedly reduced anti-pig antibody response and no Galα(1,3)Gal elicited antibody response. There was a mild cellular infiltrate that was confined to the graft periphery, conferring resistance to delayed rejection. In a cardiac transplant model, Chen et al. (C.G. Chen et al. Transplantation

65:832, 1998) showed that α1,2FT transgenic and α1,3GT knockout tissue were both protected against hyperacute rejection.

It can therefore be anticipated that the use of α1,3GT knockout tissue (having an even lower level of Galα(1,3)Gal antigen), in combination with standard immunosuppressive regimens, will enable engraftment and survival of therapeutically important grafts according to the claimed invention.

Rejection under 35 USC § 101

Claims 1-6 and 33-37 stand rejected under § 101 as claiming subject matter without a credible asserted or a well-established utility.

In making a rejection under this Section, the Examiner referred to the Office's Utility Guidelines[10] as tying the utility requirement of § 101 to the enablement requirement of § 112 ¶ 1. In fact, the standard referred to is that inventions lacking utility are not considered enabled — not *vice versa.* All the arguments made under this heading in the Office Actions are essentially enablement rejections — again, because of the lack of a full working example in the specification.

The courts have repeatedly found that the mere identification of a pharmacological activity relevant to an asserted use provides an immediate benefit to the public, thereby satisfying the utility requirement.

> Knowledge of the pharmacological activity of any compound is obviously beneficial to the public. . . . Since it is crucial to provide researchers with an incentive to disclose pharmacological activities in as many compounds as possible, we conclude that adequate proof of any such activity constitutes a showing of practical utility. *Nelson v. Bowler*, 206 USPQ 881, 883 (CCPA 1980).

This is true even if the pharmaceutical agent is in a very early stage of development. *Cross v. Iizuka,* 224 USPQ 739 (Fed. Cir. 1985).

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[10] Examination Guidelines for Utility Requirement, B2(2), Federal Register Vol 66(4), published January 5, 2001. Referred to in the Office Action dated December 16, 2004, page 4.

As indicated throughout the specification, ovine tissue devoid of antibody-detectable Galα(1,3)Gal determinants are useful and under development in a number of laboratories for use in xenotransplantation.

The Office Action indicates that the claimed invention has no utility with respect to heterozygous α1,3GT knockout animals and tissues, because there is no phenotypic difference from normal ovine animals and tissues. However, the specification teaches on page 41, lines 14-17 that heterozygous α1,3GT knockout animals have utility for making homozygous knockout animals by cross-breeding. The use of heterozygous knockout animals to breed a homozygous knockout animal is covered in Claim 13, which has not been rejected under § 101. Crossbreeding of heterozygous knockouts has been used successfully to generate α1,3GT knockouts in mice (U.S. Patent No. 5,849,991).

Furthermore, the specification teaches on page 41, lines 17-20 that tissue from heterozygous α1,3GT knockout animals (both birthed animals and fetuses) have utility for targeting the second allele, thereby obtaining homozygous α1,3GT knockout cells. This in turn can be used for nuclear transfer for production of homozygous knockout animals. Second allele targeting and recloning has been used successfully to generate α1,3GT knockouts in pigs (Phelps et al., Science 299, 411-414, 2003; Kolber-Simonds et al., Proc. Natl. Acad. Sci. USA 101:7335, 2004).

Thus, there are several asserted and credible utilities of α1,3GT heterozygous cells and animals that meet the requirements of § 101.

Appellants respectfully requests that rejection of all claims under examination be reversed, and that the application be allowed and sent to the issue branch without further delay.

If any fees are necessary for the submission of this paper, please charge our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: May 9, 2006

By: _____

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## VIII. CLAIMS APPENDIX

1. Ovine tissue devoid of antibody-detectable Gal$\alpha$(1,3)Gal determinants.

2. The tissue of claim 1, which is selected from the group consisting of lung tissue, kidney tissue, liver tissue, cardiac tissue, pancreatic tissue, and ocular tissue.

3. Isolated ovine cell or tissue that expresses glycosyl transferase enzymes but does not detectably express $\alpha$(1,3)galactosyltransferase ($\alpha$1,3GT).

4. An ovine cell which is heterozygous or homozygous for inactivation of an $\alpha$1,3GT gene.

5. The cell of claim 4, produced by transfer of a nucleus from a donor cell heterozygous or homozygous for inactivation of an $\alpha$1,3GT gene, to an enucleated recipient cell.

6. An ovine animal that is homozygous for inactivation of an $\alpha$1,3GT gene.

7. *(Withdrawn)* A polynucleotide construct effective for inactivating an $\alpha$1,3GT gene in an ovine cell.

8 to 12. *Cancelled*

13. A method for producing an ovine that is homozygous for inactivation of an $\alpha$1,3GT gene, comprising providing an ovine embryo of cells according to claim 4, engrafting the embryo into the uterus of a female, birthing an ovine with an inactivated $\alpha$1,3GT gene from the engrafted female, and if the birthed ovine has the $\alpha$1,3GT gene inactivated on only one allele, then mating it with another ovine with

an inactivated α1,3GT gene, thereby producing an ovine that is homozygous for inactivation of the α1,3GT gene.

14. A method for producing an isolated ovine cell that expresses glycosyl transferase enzymes but does not detectably express α1,3GT, comprising isolating the cell from an ovine homozygous for inactivation of an α1,3GT gene according to claim 6.

15. A method for producing ovine tissue devoid of antibody-detectable Galα(1,3)Gal determinants, comprising harvesting the tissue from an ovine homozygous for inactivation of an α1,3GT gene according to claim 6.

16. A method of xenotransplantation, comprising transplanting tissue devoid of antibody-detectable Galα(1,3)Gal determinants according to claim 1 into a mammal having circulating antibody against Galα(1,3)Gal determinants.

17. *(Withdrawn)* An isolated polynucleotide that comprises a sequence of at least 30 consecutive nucleotides with at least one of the following properties:

a) it is contained in SEQ. ID NO:1 or any of SEQ. ID NOs:14 to 25, but not in any of SEQ. ID NOs: 3, 5, 7, 9, 11, and 13;

b) it is contained in phage **B**, **C** and **G** deposited under Accession Nos. NCIMB 41056, 41059, 41060, and 41061; but not in λ-phage or any of SEQ. ID NOs: 3, 5, 7, 9, 11, and 13; or

c) it hybridizes under stringent conditions to a polynucleotide with the sequence in SEQ. ID NO:1 or any of SEQ. ID NOs:14 to 25, but not to a polynucleotide with the sequence in any of SEQ. ID NOs: 3, 5, 7, 9, 11, and 13

18 to 21. *Cancelled*

22. *(Withdrawn)* An isolated polypeptide that comprises a sequence of at least 10 consecutive amino acids with at least one of the following properties:

a) it is contained in SEQ. ID NO:2 but not in any of SEQ. ID NOs: 4, 6, 8, 10, and 12;

b) it is encoded in phage **B**, **C** and **G** deposited under Accession Nos. NCIMB 41056, 41059, 41060, and 41061., but not encoded in λ-phage or present in any of SEQ. ID NOs: 4, 6, 8, 10, and 12; or

c) it is at least 80% identical to 15 consecutive amino acids contained in SEQ. ID NO:2, wherein said sequence is not present in any of SEQ. ID NOs: 4, 6, 8, 10, and 12

23 to 26. *Cancelled*

27. *(Withdrawn)* An isolated polynucleotide comprising a sequence encoding a polypeptide according to claim 22.

28. *(Withdrawn)* An isolated polyclonal antibody or a monoclonal antibody that binds specifically to a polypeptide with the sequence SEQ. ID NO:2 but not to a peptide with the sequence present in any of SEQ. ID NOs: 4, 6, 8, or 10.

29. *(Withdrawn)* An assay method for determining α1,3GT expression by a cell, comprising contacting a polynucleotide according to claim 17 with the cell or with mRNA or cDNA obtained from the cell, detecting any hybrids that form as a result, and correlating presence of the hybrids with expression of α1,3GT by the cell.

30. *(Withdrawn)* A method for producing the antibody specific for sheep α1,3GT, comprising immunizing an animal or contacting an immunocompetent particle with a polypeptide according to claim 22.

31. *(Withdrawn)* A method for preparing a Galα(1,3)Gal determinant, comprising contacting a galactose acceptor saccharide with the polypeptide of claim 26 in the presence of UDP-galactose.

32. *(Withdrawn)* An assay method for determining α1,3GT in a sample, comprising preparing a reaction mixture comprising the sample and an antibody according to claim 28 under conditions that permit the antibody to complex with α1,3GT, and correlating any complex formed with the presence or amount of α1,3GT in the sample.

33. The cell of claim 4, which is a fibroblast.

34. The cell of claim 4, which is a kidney cell.

35. The cell of claim 4, which is a hepatocyte.

36. The cell of claim 4, which is a cardiac cell.

37. The cell of claim 4, which is an islet cell.

## IX. EVIDENCE APPENDIX

The following evidence is relied upon in Appellants' Brief. The evidence cited below was entered in the record by the Examiner as indicated below. A copy of each is attached.

1. 35 USC § 1.132 Declaration signed by Ian Wilmut, Ph.D., O.B.E., F.R.S., filed in the application on September 23, 2004.

2. U.S. Patent 5,589,369. Submitted in IDS 10/30/00; considered by Examiner 11/15/01.

3. U.S. Patent 5,849,991. Submitted in IDS 10/30/00; considered by Examiner 11/15/01.

4. U.S. Patent 5,821,117. Submitted in IDS 10/30/00; considered by Examiner 11/15/01.

5. U.S. Patent 6,147,276. Submitted in IDS 12/6/01; considered by Examiner 1/23/03.

6. U.S. Patent 6,252,133. Submitted in IDS 12/6/01; considered by Examiner 1/23/03.

7. Denning et al., Nature Biotechnology 19:559, 2001. Cited by Examiner in PTO-892 as part of Paper No. 5.

8. Phelps et al., Science 299, 411-414, 2003. Submitted in IDS 3/31/03; considered by Examiner 4/15/03.

9. Kolber-Simonds et al., Proc. Natl. Acad. Sci. USA 101:7335, 2004. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

10. Uchida et al., Transgenic Research 10:577, 2001. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

11. Bondoli et al., Molec. Repro. Dev. 60:189, 2001. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

12. Lai et al., Molec. Repro. Dev. 62:300, 2002. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

13. McCreath et al., Nature 405:1004, 2000. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

14. Lai et al., Science 295:1089, 2002. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

15. Dai et al., Nature Biotech 20:251, 2002. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

16. Chong et al., Transpl Immunol 8:129-37, 2000. Submitted in IDS 12/3/02; considered by Examiner 4/15/03.

17. Linder et al., Lab. Anim. 30:34, 2001. Cited by Examiner in PTO-892 as part of Paper No. 7.

18. Schnieke et al., Science 278:2130, 1997. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

19. Cibelli et al., Science 280:1256, 1998. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

20. Kuroiwa et al., Nature Genetics 36:775, 2004. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

21. Ramsoondar et al., Biol. Reprod. 69:437, 2003. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

22. Sendai et al., Transplantation 76:900, 2003. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

23. Dor et al., Transplantation 78:15, 2004. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

24. Stinson et al., J. Thorac. Cardiovasc. Surg. 73:54, 1977. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

25. Angell et al., Ann. Thorac. Surg. 28:537, 1979. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

26. Costa et al., FASEB J. 17:109, 2003. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

27. C.G. Chen et al., Transplantation 65:832, 1998. Submitted in IDS 8/17/04; considered by Examiner 12/1/04.

X. Related Proceedings Appendix to Appeal Brief Under Rule 41.37(c)(1)(x)

None

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: J. Clark & C. Denning

Filing Date: June 13, 2000

Serial No: 09/593,316

Docket: 730/002

Title: ANIMAL TISSUE FOR XENOTRANSPLANTATION

Art Unit: 1632

Examiner: Qian J. Li, Ph.D.

**DECLARATION UNDER 37 CFR § 1.132**
**BY IAN WILMUT, Ph.D., O.B.E., F.R.S.**

Commissioner for Patents
Alexandria VA 22313

Dear Sir:

I, IAN WILMUT, do hereby declare as follows:

I am head of the Department of Gene Function and Development at the Roslin Institute in Midlothian, Scotland. The group headed by Keith Campbell and myself cloned Dolly the Sheep — the first mammal to be cloned from an adult cell. The methods we used are described in U.S. Patent Nos. 6,147,276; 6,252,133; and 6,525,243.

I have reviewed the Patent Application by John Clark and Chris Denning referred to at the top of this Declaration. I understand the Examiner has questioned whether genetically qualified animals can be made according to the Campbell and Wilmut method, and whether a homozygous α(1,3)galactosyltransferase knockout sheep can be made using the gene sequence information provided in this patent application.

After the sheep α1,3GT gene was isolated as described in the application, Dr. Clark and Dr. Denning turned their attention to making α1,3GT knockout sheep. Limited resources were available to pursue the project, and the project was not completed. Preliminary results were reported in the research article published by C. Denning et al., Nature Biotech. 19:559, 2004, for which I am a coauthor.

The paper reports that heterozygous α1,3GT knockout cells were produced as donor cells for cloning by nuclear transfer, but no fetus survived the full term of pregnancy. When the longest lived fetus was autopsied, we found abnormalities around the blood vessels in the lung, which were apparently fatal.

Accompanying this Declaration is an article which I coauthored with Susan Rhind et al. (Nature Biotech. 21:744, 2003). The article explains that lung abnormalities of this kind are seen in failed neonatal cloned sheep more often than they are seen in normal sheep pregnancies. Amongst the animals listed in Table 1, animals 1, 3, 4, and 5 were cloned from knockout cells; animal 6 was cloned from a cell containing a randomly integrated transgene, and animals 2, 7, and 8 were cloned from cells without any genetic modification. Lung abnormalities were seen in cases 1, 3, 5, 6, and 7, which means that the abnormality is not attributable to the use of genetically modified cells, but is an artifact of the cloning process in general. We believe the lung abnormalities are due to incomplete reprogramming when the nucleus of the donor cell is transferred to the recipient oocyte during cloning.

The Denning article shows that there was a frequency of failed pregnancies whether they contained a α1,3GT knockout (Table 2, lines 3C6 and 5E1), a randomly integrated gene (4H2), a knockout of the PrP gene (YH6), or were cloned from unaltered cells (7G65F4). Failure of the α1,3GT knockouts was not attributable to the genetic modification, which has no known relationship to the viability of smooth muscle cells. Rather, it reflects the rate of failure in this series of experiments, irrespective of what genetic modifications were made. As explained in the article, we attribute the rate of failure to the number of doublings of the cells in tissue culture.

There is no reason why genetically modified animals cannot be made according to the method that Keith Campbell described in our patent disclosures. Using donor cells that have not undergone extensive culturing, or that have a long replicative capacity may improve the frequency of successful cloning. But it is only the frequency that is affected, not the ultimate efficacy. It is my belief that cultured cell lines such as those used by Denning et al. will successfully generate cloned animals after sufficient persistence.

This is illustrated by the successful cloning of heterozygous and homozygous knockouts and transgenic animals by other laboratories, using the Campbell and Wilmut technique. For example, Phelps et al. (Science 299, 411-414, 2003) and Kolber-Simonds et al. (Proc. Natl. Acad. Sci. USA 101:7335, 2004) have both cloned α1,3GT knockout pigs. Kuroiwa et al. (Nature Genetics 36:775, 2004) cloned cattle that contain homozygous knockouts of both the IgM μ-chain gene, and the PrP gene.

The cloning method used by all these groups is the same as described by Keith Campbell and myself in our U.S. Patents. There is no modification to any aspect of our method — selection of the oocyte, transfer of the nucleus, activation of the combined cell, or implantation into the surrogate female — that is needed for the method to work when the donor cell has been genetically modified.

The patent application by Denning and Clark provides the sheep α1,3GT gene, and describes the making of targeting vectors, and knockout cells. Making α1,3GT knockout sheep from the sheep α1,3GT gene should be no more difficult than making α1,3GT knockout pigs from the pig α1,3GT gene.

PATENT
09/593,316
Docket 730/002

I hereby declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Sept 9, 2004
Date

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# Deletion of the α(1,3)galactosyl transferase (*GGTA1*) gene and the prion protein (*PrP*) gene in sheep

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Nuclear transfer offers a cell-based route for producing precise genetic modifications in a range of animal species. Using sheep, we report reproducible targeted gene deletion at two independent loci in fetal fibroblasts. Vital regions were deleted from the α(1,3)galactosyl transferase (*GGTA1*) gene, which may account for the hyperacute rejection of xenografted organs, and from the prion protein (*PrP*) gene, which is directly associated with spongiform encephalopathies in humans and animals. Reconstructed embryos were prepared using cultures of targeted or nontargeted donor cells. Eight pregnancies were maintained to term and four $PrP^{-/+}$ lambs were born. Although three of these perished soon after birth, one survived for 12 days. These data show that lambs carrying targeted gene deletions can be generated by nuclear transfer.



Gene targeting in embryonic stem (ES) cells is a powerful tool for modifying the genome of mice[1]. In other species, ES cells that contribute to the germline are not available, limiting widespread use of the technique. With the development of nuclear transfer in livestock species[2–4], genetically engineered somatic cells can be used to modify the genome. Previously, transgenic sheep expressing the human Factor IX gene in the mammary gland were produced by this route after random integration of the transgene into donor cell nuclei[4]. More recently, viable animals have been produced after gene targeting was used to precisely insert human α1-antitrypsin (AAT) sequences into the *COL1A1* locus[5], although the insertion site was specifically selected so as not to disrupt type 1 collagen protein function or expression. Targeted gene disruption is essential when complete deletion of gene function is required.

Animals potentially offer an alternative source of tissue for transplantation. A major barrier to successful xenotransplantation is presented by preformed antibodies that recognize the disaccharide galactose-α(1,3)-galactose, leading to hyperacute rejection[6]. Synthesis of galactose-α(1,3)-galactose is catalyzed by the enzyme α(1,3)galactosyl transferase, which is present in all organisms except catarrhines (Old World monkeys, apes, and humans). The hypothesis that deletion of this gene from the germline of donor species may eliminate a substantial component of hyperacute rejection needs to be tested in a large-animal model. Although the pig has been highlighted as the ideal choice for xenotransplantation, concerns have been raised about anatomical incompatibilities with humans[7] and the retroviral load of the porcine genome[8]. Sheep lacking α(1,3)galactosyl transferase could be used to determine the importance of galactose-α(1,3)-galactose in graft rejection, to develop immunosuppression regimes, and to provide tissues for xenotransplantation.

Prions, encoded by the *PrP* gene, are a novel form of infectious agent that cause spongiform encephalopathies in humans and animals[9]. Prions have assumed tremendous importance because of the bovine spongiform encephalopathy (BSE) epidemic and the concern that there has been cross-species transmission to humans, resulting in a new and highly lethal form of Creutzfeld–Jacob disease. Experiments with *PrP* gene knockout mice have shown that these animals do not replicate the prion gene and are resistant to scrapie[10,11]. Because sheep, and particularly cattle, have functional *PrP* genes and are used to produce biomedical products such as gelatin, collagen, and, increasingly, human proteins after genetic modification, it may be appropriate to produce prion-resistant populations.

We therefore selected the *GGTA1* and *PrP* genes as candidates for deletion from sheep. In addition, genetically engineered mice without one or the other of these genes show no gross deleterious effects[12–14], indicating that they would be appropriate targets to develop gene disruption technology in livestock. Here we report use of nuclear transfer to produce sheep that have targeted gene deletions.

## Results and discussion

The ovine *PrP* gene has previously been cloned and characterized. Three exons span 21 kilobases of genomic DNA, with the 770 base pair coding region contained entirely within the final exon[15]. Comparable data for the *GGTA1* gene were not available, although the coding sequence was known for other species[16,17]. Using primers that functioned across species in a reverse transcriptase–polymerase chain reaction (RT-PCR), we isolated an 1,110 base pair ovine *GGTA1* complementary DNA (cDNA), which showed 83% and 95% homology to murine and bovine sequences, respectively. A 193 base pair 5′-untranslated region was extended by rapid amplification of cDNA ends (RACE) PCR, although it appears to be truncated compared with the corresponding region in the mouse gene.

To generate targeting vectors, we used *GGTA1* or *PrP* DNA probes to screen a genomic library prepared from tissue culture cells derived from a day 35 Black Welsh fetus. The coding exons of the *GGTA1* gene span ~20 kilobases of genomic DNA and were designated 4 to 9 (Fig. 1), because translation initiation occurs in exon 4 of the well-characterized mouse gene[16]. The *PrP*-hybridizing phage were analyzed and had the same sequence and restriction pattern as in the published data[15].

The *GGTA1* and *PrP* genes are expressed in fetal fibroblasts (data not shown), permitting use of the promoter trap targeting strategy[18]. In the vectors constructed, the neomycin phosphotransferase (*neo*) gene was

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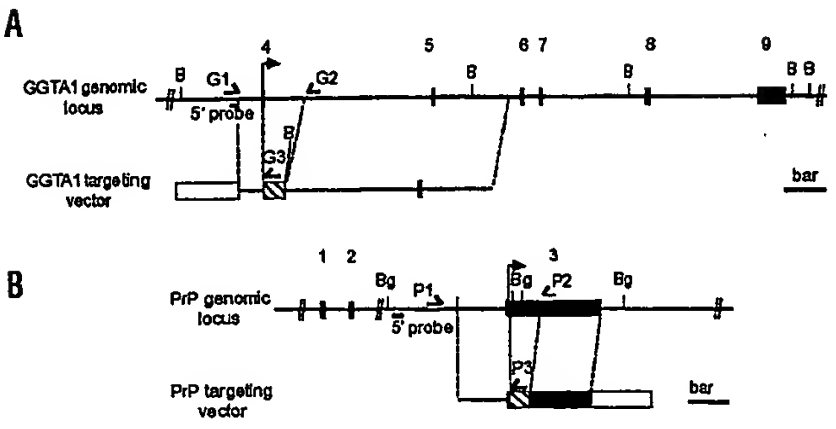


**Figure 1.** Organization of the genomic loci of ovine (A) *GGTA1* or (B) *PrP* genes and the promoterless targeting vectors used for disruption. Numbering of the exons in *GGTA1* is based on the mouse; translation initiates in exon 4 and terminates in exon 9. Targeting deletes exon 4 and 1.4 kb of intron 4, and a *Bam*HI site (labeled B) is inserted. The coding sequence of *PrP* is entirely within exon 3; targeting deletes this region and two *Bgl*I sites (labeled Bg). Arrows indicate translation initiation sites. Black boxes represent exons, hatched boxes represent *neo*-pA sequence, and open box represents pBlueScript sequence. Location of PCR primers (*GGTA1* uses G1/2 and G1/3; *PrP* uses P1/2 and P1/3) and the 5′ external probes for Southern blot analysis are shown. Scale bar represents 2 kb.

placed directly adjacent to the initiation codon of the target genes (Fig. 1). The *PrP* targeting vector does not delete the splice acceptor site of exon 3, a component of the gene that must be retained to generate knockout mice that are clinically healthy and do not have a grossly aberrant phenotype[14]. Linearized *GGTA1* or *PrP* vectors (10 μg) were transfected into early-passage BW6F2 karotypically normal male (54XY) cells. After 12 days of G418 selection, 877 and 533 colonies had grown in the *GGTA1* and *PrP* experiments, respectively (Table 1).

Initially, we used two independent PCR reactions to detect targeting events for each construct. Using this strategy, we demonstrated that 1.1% (10) or 10.3% (55) of the *GGTA1* or *PrP* BW6F2 neomycin-resistant ($neo^R$) colonies contained correctly targeted cells (Table 1). However, in terms of selecting a clonal targeted population with a stable karyotype that could be expanded for use in several nuclear transfer (NT) experiments, only one colony ($PrP^{-/+}$, termed YH6) was suitable (Table 1; Fig. 2B, lane 1). Many targeted colonies also contained nontargeted cells, as indicated by the greater intensity of the PCR band from the nontargeted allele compared with that of the targeted allele. More importantly, a substantial number of colonies (4/5 *PrP* and 8/8 *GGTA1*) with only targeted cells senesced before they could be prepared for nuclear transfer (Table 1). The high attrition rate of targeted clonal populations suitable for nuclear transfer (Table 1) represents one of the major hurdles of gene targeting in primary somatic cells.

Targeting experiments at the *GGTA1* locus were continued using a different primary cell culture, 7G65F4, isolated from a Finn Dorset fetus. These cells were in culture for 6 days before electroporation, compared with ~14 days for the BW6F2 cells used before. Targeting events were detected at a frequency of 6.2% (35 of 568). Ultimately, two *GGTA1*-targeted colonies (3C6 and 5E1) suitable for nuclear transfer were isolated (Table 1; Fig. 2A, lanes 1 and 4).

We have shown targeting frequencies in $neo^R$ clones of 1.1 and 6.2% for the *GGTA1* locus and of 10.3% for the *PrP* locus. These are upper estimates, as the data include a substantial proportion of mixed clones, but correspond to an overall targeting frequency of 1–10 per $10^6$ cells. Recently McCreath and colleagues[5] reported targeting efficiencies of 7.1, 13.8, and 65.7% in the ovine *COL1A1* locus in Poll Dorset fetal fibroblasts. The high average efficiency in these experiments may be attributable to high endogenous expression or intrinsic recombinogenic activity at this locus. Alternatively, the vector used by these workers had contiguous regions of homology with the chromosomal locus and did not delete any of the *COL1A1* gene. By contrast, to ensure effective disruption of the *GGTA1* and *PrP* genes, we deleted endogenous coding sequence with *neo*-polyA sequence using noncontiguous regions of homology.

Targeted (3C6, 5E1, or YH6) and control cells (4H2, with a random integration of the *GGTA1* targeting vector: Fig. 2A, lane 7; 7G65F4, nontransfected parental line) were prepared for nuclear transfer by culturing in low- (0.5%) serum medium for three to five days. Donor cells were fused to enucleated Poll Dorset oocytes, as described[2]. A total of 120 morulae or blastocysts were transferred to 78 Finn Dorset final recipients, which produced 39 pregnancies at day 35. The oldest *GGTA1*-targeted fetuses died *in utero* at 118 and 130 days (term 148 days). Eight pregnancies were maintained to term (two 7G65F4, one 4H2, five YH6), resulting in four live births derived from the *PrP*-deleted line, YH6. Three of these lambs perished soon after birth. One lived for 12 days (Table 2; Fig. 3) but was euthanized after developing dyspnea due to pulmonary hypertension and right-sided heart failure, common abnormalities in cloned sheep.

The high incidence of mortality reported here may indicate that genetic modification or prolonged culture is detrimental to development. Although comparison of the developmental stages revealed similar efficiencies of progression from targeted cells, nontargeted cells with random integration, and untransfected cultures (blastocyst, 10–31%; day 35, 3.3–6.7%; day 60, 0–4.4%, referenced to embryos transferred or cultured; Table 2), we observed a high incidence of mortality at and soon after birth. This contrasts with other studies using unmodified, early-passage sheep cells[2–4]. However, it is consistent with a recent report[5] of gene insertion in sheep; although two targeted animals survived beyond three months, there was a high incidence of perinatal and postnatal mortality. Thus prolonged culture, in combination with the stringent selection required for somatic gene targeting, may produce cell lines that are less competent at producing viable clones.

When possible, autopsies were performed. The range of abnormalities found was consistent among the different groups. The predominant findings were hydroallantois, distention of the liver caused by congestion (suggestive of cardiac insufficiency), insufficient placentation indicated by reduced numbers and size of cotyledons, and kidney dysplasia manifested by enlarged renal pelvis with narrowed cortex and medulla. All these defects have been described in other nuclear transfer experiments with nontrans-

**Table 1. Efficiency of gene targeting in ovine somatic cells**

| Parental primary culture | Target gene | G418-resistant colonies | Total targeting events detected[a] | Mixed colonies[b] | Senesced[c] | Unstable[d] karyotype | Targeted colonies suitable for NT |
|---|---|---|---|---|---|---|---|
| BW6F2 | *GGTA1* | 877 | 10 | 2 | 8 | 0 | 0 |
| BW6F2 | *PrP* | 533 | 55 | 50 | 4 | 0 | 1 (YH6) |
| 7G65F4 | *GGTA1* | 568 | 35 | 17 | 15 | 1 | 2 (3C6, 5E1) |

[a]Total number of targeting events detected by the initial PCR screens.
[b]Colonies were scored as mixed when the amplified band from the nontargeted locus was more intense than the targeted locus in the second PCR screen.
[c]Colonies were scored as senesced when cell numbers could not be seen to increase after seven days.
[d]The normal karyotype of these cells was 54XY.

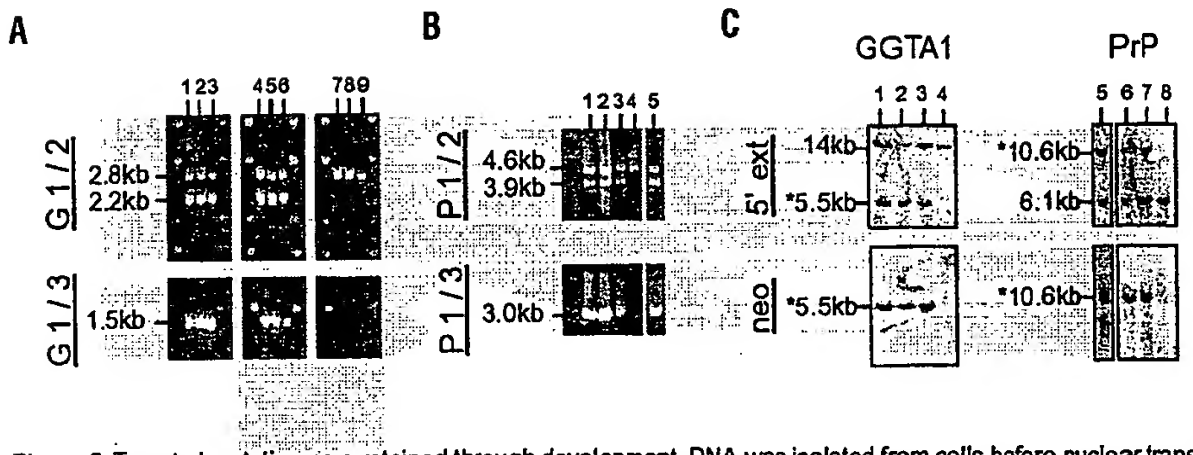


Figure 2. Targeted mutations are retained through development. DNA was isolated from cells before nuclear transfer or from derived fetuses, then analyzed by PCR and Southern blot. Samples with a targeted allele are indicated by an asterisk (*). See Figure 1 for location of primers and probes. (A) *GGTA1* PCR. Lanes 1, 2, and 3 show 3C6* cells, and fetuses at day 85* and day 118*. Lanes 4, 5, and 6 show 5E1* cells, and fetuses at day 49* and day 49*. Lanes 7, 8, and 9 show 4H2 cells, and fetuses at day 49 and day 148. (B) *PrP* PCR. Lane 4 shows nontargeted parental cells. Lane 1 shows YH6* cells. Lanes 2 and 3 show lambs carried to term*. Lane 5 shows the targeted lamb that survived to 12 days*. (C) Southern blot analysis. *GGTA1* samples were digested with *Bam*HI. The targeted allele hybridizes with the 5′ and neo probes; lanes 1, 2, and 3 show samples from fetuses at day 118*, day 49*, and day 49*. Lane 4 shows a nontargeted sample. *PrP* samples were digested with *Bgl*I. Lane 5 shows the targeted lamb that survived to 12 days*. Lanes 6 and 7 show samples from fetuses at term*. Lane 8 shows a nontargeted sample.

Figure 3. $PrP^{-/+}$ lamb photographed at six days postpartum.

fected cells[2,5,19,20]. We did not expect abnormal phenotypes as a direct result of the gene disruption because we modified only one allele at a dominant locus. Furthermore, null mice for *GGTA1* or *PrP* are healthy[12–14].

Tissue was recovered from fetuses and lambs for both PCR and Southern blot analysis. Data are shown for fetuses ranging from day 49 to 148 (term) of pregnancy. The two PCR screens for each locus revealed patterns consistent with targeting (Fig. 2) in all the samples that were recovered. In Southern blot analyses, both 5′ (external) and neo coding sequence (internal) probes hybridized to restriction fragments of the correct size. The location of probes and restriction sites is shown in Figure 1; representative Southern blots are shown in Figure 2. These data show that lambs carrying targeted gene deletions can be generated by nuclear transfer.

Our results, together with the recent report of sequence insertion at the ovine *COL1A1* locus[5], indicate that targeted homologous recombination has been demonstrated at three independent loci in cells derived from different breeds of sheep. This suggests that the technology can be used to disrupt many different genes in the ovine genome. We found, however, that the number of targeted clones suitable for nuclear transfer was low. A major barrier was that many of the clonal populations reached proliferative senescence. The bulk populations of the primary cultures we used divide ~100 times before senescing, a large excess compared to the estimated 45 doublings required for targeting and preparation for nuclear transfer[21]. A likely explanation is that there is considerable heterogeneity of life span in the culture, with many of the selected colonies having a life span considerably shorter than 100 doublings.

The death of the targeted fetuses and lambs emphasizes the need to improve the efficiency of the technology. Once this is achieved, effective ablation of gene function will usually require both alleles to be disrupted. Given the limited proliferative capacity of cells currently used in nuclear transfer, achieving this from a single clonal population will be difficult. Alternatively, conventional breeding could be used with animals surviving to reproductive maturity. However, this would take a minimum of 18 months in sheep, even if the modification were introduced simultaneously into male and female cells and the cloned animals interbred. A different approach would be to clone by nuclear transfer from the cells in which the first allele has been targeted, re-isolate cell lines from the cloned fetal material, and then target the second locus in these cells[22,23]. Ultimately, however, the fastest route to multiple genetic changes would be to extend the window to achieve targeting, either by increasing the overall efficiency of targeting or by using cells with an extended life span that still retain their totipotency for nuclear transfer.

**Table 2. Nuclear transfer from gene-targeted primary cells[a]**

| Stage of nuclear transfer | Cells used for nuclear transfer | | | | |
|---|---|---|---|---|---|
| | 3C6 | 5E1 | 4H2 | 7G65F4 | YH6 |
| Embryos transferred into temporary recipients[b] (*in vitro* cultured) | 87 (25) | 0 (30) | 92 (31) | 55 (71) | 273 (181) |
| Embryos recovered from temporary recipients | 85 | – | 62 | 55 | 214 |
| Morula or blastocyst[b]: *in vivo* (*in vitro*) | 18 (7) | 0 (3) | 19 (8) | 12 (27) | 44 (3) |
| Embryos transferred to final recipients | 18 | 3 | 23 | 33 | 43 |
| Final recipients | 12 | 3 | 17 | 18 | 28 |
| Fetuses at day 35 | 7 | 2 | 4 | 8 | 18 |
| Fetuses at day 60 | 5 | 0 | 2 | 5 | 8 |
| Lambs at birth: live (dead) | 0 | 0 | 0 (1) | 0 (2) | 3 (1) |
| Lambs alive at one week | 0 | 0 | 0 | 0 | 1 |

[a]Data are shown for various cultures: 3C6 and 5E1 (*GGTA1* correctly targeted), 4H2 (randomly integrated *GGTA1* targeting vector), and 7G65F4 (untransfected cells) were of Finn Dorset origin; YH6 (*PrP* correctly targeted) was of Black Welsh origin. Poll Dorset oocytes were used as recipient cytoplasts throughout.
[b]Reconstructed embryos were transferred to temporary recipients, unless the number of oocytes recovered was low or fusion could not be seen and *in vitro* culture (additional embryos shown in parentheses) was adopted.

## Experimental protocol

**Isolation, culture, and transfection of primary fibroblasts.** Black Welsh (BW6F2) or Finn Dorset (7G65F4) fibroblasts were recovered from day 35 fetuses as described[2]. Cells were cultured in BHK21 medium (Sigma, St. Louis, MO) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids (Life Technologies, Rockville, MD), and 10% FCS (Globe Farm, Gilford, Surrey, UK) in a humidified environment with 5% $CO_2$. Linearized targeting construct (10 μg) was electroporated to passage one 7G65F4 (125 μF/350 V, *GGTA1*) or passage six BW6F2 (250 μF/400 V, *PrP*) cells ($5 \times 10^6$), which were then seeded in 96-well plates ($2.5 \times 10^3$ cells/well). G418 selection (400 μg/ml) was applied after 24 h. At subconfluence, resistant colonies were replica plated to two 96-well plates for DNA analysis or cryopreservation.

**Targeting constructs.** Promoterless vectors, with *neo-pA* sequence (Stratagene, La Jolla, CA) adjacent to the endogenous gene start codon, were used to target the *GGTA1* and *PrP* loci. The *GGTA1* vector was constructed by amplifying a truncated left arm (300 bp; using primers 199001, 5′-ACGTGGCTCCAAGAATTCTCCAGGCAAGAGTACTGG-3′ and 199006, 5′-CATCTTGTTCAATGGCCGATCCCATTATTTTCTCCTGGGAAAAGAAAAG-3′, with tail complementary to the start of neo coding sequence) and neo-polyA sequence (using primers 199005, 5′-CTTTTCTTTTCCCAGGAGAAAATAATGGGATCGGCCATTGAACAAGATG-3′, with tail complementary to left arm, and 199004, 5′-CAGGTCGACGGATCCGAACAAAC-3′). These fragments were used to prime from each other to give a 1.2 kb fusion product. This was ligated to intron 3 sequence (1 kb *Eco*RV-*Eco*RI fragment), to extend the left arm, and to ~9 kb (*Eco*RV partial digest–*Not*I) of 3′ sequence to create the right arm.

The *PrP* vector was constructed by amplifying the left arm (2.4 kb; using primers prp6F, 5′-CCGAGCTCGCCAATTTCATGGCTGCAGTCACC-3′; and prp7R, 5′-CGATCCCATGATGACTTCTCTGCAAAATAAAG-3′, with tail complementary to the start of neo coding sequence) and neo-polyA sequence (using primers prp10F, 5′-GAGAAGTCATCATGGGATCGGCCATTGAACA-3′, with tail complementary to left arm; and prp8R, 5′-TGCAGGTCGACGGATCCGAA-3′). These fragments were used to prime from each other to give a 3.3 kb fusion product, which was ligated to a 3 kb *Kpn*I fragment to complete the vector.

The *GGTA1 or PrP* vectors were linearized with *Not*I or *Sac*I, respectively, before electroporation.

**DNA analysis.** Drug-resistant colonies were screened for targeting events by PCR. DNA was isolated in 96-well plates by overnight lysis (50 mM Tris, pH 8, 20 mM ethylenediamine tetraacetate, 100 mM NaCl, 0.3% sodium dodecyl sulfate, 10 mg/ml proteinase K), then isopropanol precipitated, and pellets were resuspended in 50 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8). Amplification was performed using Roche Expand HiFi kit, with 1 μl DNA template. Primer locations are indicated in Figure 2: G1 (5′-CAGCTGTGTGGGTATGGGAGGG-3′); G2 (5′-CTGAACTGAATGTTTATCCAGGCCATC–3′); G3 / P3 (5′-AGCCGATTGTCTGTTGTGCCCAGTCAT–3′); PR1 (5′-TTCAGTCGCTCTGTTGTGTC CCA-3′); P2 (5′-AGCATCCCTC CTGCCTTCAG TTCTTC-3′). Cycling conditions for *GGTA1* were 94°C, 2 min/94°C, 30 s / 65°C, 30 s / 68°C, 2.5 min (10 cycles); 94°C, 30 s / 65°C, 30 s / 68°C, 2.5 min + 5 s per cycle (20 cycles); 68°C, 7 min. For *PrP* the elongation phase was increased to 4 min. Products were analyzed by agarose gel electrophoresis.

For Southern blot analysis, genomic DNA was digested with *Bam*HI or *Bgl*I (*GGTA1* or *PrP*, respectively) and blotted to Ambion bright star membrane according to manufacturer's instructions. Diagnostic bands were detected using Ultrahyb (Ambion, Austin, TX) with DNA probes corresponding to *neo* sequence (Stratagene), *GGTA1* 5′ probe (a 100 bp fragment was produced by PCR using forward [CAGCTGTGTGGGTATGGGAGGG] and reverse [CTAACTACGTGCTCCGCCGTTCA] primers) or *PrP 5′* probe (corresponding to 16,701–17,151 bp of accession no. U67922, Entrez, NCBI).

**Nuclear transfer.** Somatic cell nuclear transfer was based on the method of Wilmut[2]. Oocytes were collected from superovulated Poll Dorset ewes in PBS with 1% FCS and transferred immediately to calcium-free HEPES-buffered synthetic oviduct fluid[19] (SOF) for removal of cumulus and enucleation. If necessary, cumulus was removed by pipetting in 600 IU/ml hyaluronidase. Oocytes were exposed to 5 μg/ml Hoechst 33248 and 7.5 μg/ml cytochalasin B. Sheep fetal fibroblasts were cultured for three to five days in serum-deficient medium (0.5% FCS) before use as karyoplast donors. Simultaneous fusion of donor cells and recipient oocytes, and activation of the recipient oocytes, was achieved by three consecutive 80 μs pulses of 1.25 kV/cm² in 0.3 M mannitol, 0.1 $MgCl_2$, and 0.05 mM $CaCl_2$. Reconstructed embryos were incubated for six days (*in vitro* culture) or overnight (*in vivo* culture) in SOF solution supplemented with BSA in an atmosphere consisting of 5% $O_2$, 5% $CO_2$, and 90% $N_2$ at 38°C. For *in vivo* culture, following the overnight culture, embryos were embedded in 1% agar chips in PBS and transferred into the ligated oviduct of an estrus-synchronized recipient ewe for an additional six days. Morula and blastocyst stage embryos were recovered seven days post-activation to the uteri of estrus-synchronized ewes (one to two embryos/recipient). Pregnancies were monitored using subcutaneous ultrasound scanning.

*Acknowledgments*

*The authors would like to thank J. Bowering, W. Bosma, P. Johnson, T. Ferrier, D. McGavin, B. Gasparrini, and L. Harkness for technical support, and Jane Lebkowski for reading the manuscript. The Biotechnology and Biological Sciences Research Council and the Geron Corporation provided financial support.*

Received 28 February 2001; accepted 6 April 2001

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suggest that there is space for up to three extended chains within the chamber.

Second, little difference in the rates of endoproteolytic cleavage of these disordered substrates was detected between latent 20S and active 26S proteasomes (Figs. 1 to 4) in which the status of the gate that controls entry to the central axial channel of the proteasome is closed and open respectively (Fig. 1C) (*24*, *30*). Physiological regulators of the proteasome, such as PA700 (19S cap) and PA28, increase proteasome activity in part by opening this gate, thereby increasing access of substrates to the proteasome's catalytic centers (*24*, *25*). The ability of closed, latent 20S proteasome to catalyze cleavage of these natively disordered, physiological substrates suggests they possess certain features that also promote "gating" of the proteasome (Fig. 4B), features that folded proteins lack. This mechanism suggests a potential role for the free 20S proteasome found in the absence of bound regulatory proteins in many cells (*33*). It is possible that these inherent signals could target substrates directly for 20S proteasomal degradation without the need for polyubiquitin modification.


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34. We thank S. J. Elledge (Baylor College of Medicine) for his generous gift of p21cDNA; R. Nussbaum (National Human Genome Research Institute) for α-syn cDNA; C. Wigley, R. Stidham, and S. Muallem for critical suggestions and members of our laboratories for helpful comments. This work was supported by grants from the Welch Foundation (P.J.T.), Muscular Dystrophy Association (G.N.D.), and NIH [grants DK46878 (G.N.D.) and DK49835 (P.J.T.)].


Supporting Online Material
www.sciencemag.org/cgi/content/full/1079293/DC1
Materials and Methods

10 October 2002; accepted 25 November 2002
Published online 12 December 2002;
10.1126/science.1079293
Include this information when citing this paper.

# Production of α1,3-Galactosyltransferase–Deficient Pigs

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The enzyme α1,3-galactosyltransferase (α1,3GT or GGTA1) synthesizes α1,3-galactose (α1,3Gal) epitopes (Galα1,3Galβ1,4GlcNAc-R), which are the major xenoantigens causing hyperacute rejection in pig-to-human xenotransplantation. Complete removal of α1,3Gal from pig organs is the critical step toward the success of xenotransplantation. We reported earlier the targeted disruption of one allele of the α1,3GT gene in cloned pigs. A selection procedure based on a bacterial toxin was used to select for cells in which the second allele of the gene was knocked out. Sequencing analysis demonstrated that knockout of the second allele of the α1,3GT gene was caused by a T-to-G single point mutation at the second base of exon 9, which resulted in inactivation of the α1,3GT protein. Four healthy α1,3GT double-knockout female piglets were produced by three consecutive rounds of cloning. The piglets carrying a point mutation in the α1,3GT gene hold significant value, as they would allow production of α1,3Gal-deficient pigs free of antibiotic-resistance genes and thus have the potential to make a safer product for human use.

The enzyme α1,3-galactosyltransferase (α1,3GT or GGTA1) synthesizes α1,3Gal epitopes (Galα1,3Galβ1,4GlcNAc-R) on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys (*1*). α1,3Gal epitopes are the major xenoantigens causing hyperacute rejection (HAR) in pig-to-human xenotransplantation (*2*–*4*). Many reports have also indicated that α1,3Gal epitopes are involved in acute vascular rejection (AVR) of xenografts (*4*–*6*). Piglets with α1,3GT heterozygous knockout have been cloned by our group (*7*) and another team (*8*) in the last year. To produce homozygous α1,3GT knockout piglets by natural breeding, assuming both male and female heterozygous knockout pigs are available at the same time and are fertile, is feasible but takes up to 12 months. However, by using a second-round knockout and cloning strategy, we could save up to 6 months and all cloned piglets would be α1,3GT double knockout (DKO). We have selected and enriched for α1,3GT DKO cells by using a bacterial toxin, toxin A from *Clostridium difficile*, which binds with high affinity to α1,3Gal epitopes and produces a cytotoxic effect on cells that are α1,3Gal-positive (*9*). Toxin A uses α1,3Gal epitopes as a cell

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surface receptor and causes "rounding" and lifting of the α1,3Gal-positive cells from the surface of the growth vessel (*10*, *11*).

Heterozygous α1,3GT knockout fetal fibroblasts, 657A-I11 1-6 cells, were isolated from a day-32 pregnancy as described in (*7*). To avoid using a second antibiotic-resistance gene as a selection marker, we constructed an ATG (start codon)-targeting α1,3GT knockout vector, pPL680 (*12*), which also contains a *neo* gene, to knock out the second allele of the α1,3GT gene. 657A-I11 1-6 cells were transfected by electroporation with pPL680 and selected for the α1,3Gal-negative phenotype with purified *C. difficile* toxin A (*13*). One colony (680B1) was isolated and expanded after toxin A selection. When the 680B1 cells were stained with a fluorescein-labeled α1,3Gal-specific lectin, GS-IB4, about 80% of the cells were found to be α1,3Gal-negative. The fact that fewer than 100% of the cells in the colony were negative with GS-IB4 staining indicated that this colony contained a mixture of α1,3Gal-negative and -positive cells. We used 680B1 cells for somatic cell nuclear transfer (cloning) as described in (*7*). We transferred embryos to five recipient gilts, and three initial pregnancies were established, of which only one went beyond day 35 of gestation.

To determine whether all the fetuses cloned from 680B1 cells were α1,3GT DKO, we terminated the remaining pregnancy at day 39 and recovered four normal-sized fetuses. Fibroblast cell lines (680B1-1 to B1-4) were isolated from each of these four fetuses, and fluorescence-activated cell sorting (FACS) analysis with GS-IB4 staining showed that B1-1, B1-2, and B1-4 cells were α1,3Gal-negative, whereas B1-3 cells were positive for α1,3Gal (Fig. 1). Normal human serum (NHS) contains preformed antibodies to α1,3Gal and complement proteins, which together cause rapid lysis of cells that are α1,3Gal-positive. A complement lysis assay on these cells showed that B1-1, B1-2, and B1-4 cells were resistant to lysis by NHS, but B1-3 cells were lysed by NHS at the same rate (about 40% of cells lysed) as control wild-type pig cells (Fig. 2). Analysis of genomic DNA from these fetal cells by polymerase chain reaction (PCR) and Southern blot analysis indicated that none of the three α1,3Gal-negative cell lines had the expected restriction fragment pattern predicted for targeted disruption of the second α1,3GT allele with the pPL680 knockout vector. Instead, these fetal cells appeared to have the same allele pattern (one targeted allele and one wild-type allele) as their parent 657A-I11 1-6 cells, which contained only one disrupted α1,3GT allele. Northern blot analysis of the four cell lines (B1-1 to B1-4) showed that they expressed two mRNAs of similar size to those seen in the 657A-I11 1-6 cells (fig. S1). The 3.8-kb band corresponds in size to the wild-type α1,3GT transcript and the shorter 2.5-kb band is the same size expected for the truncated transcript of the first knockout allele (fig. S1). Because of the nature of the toxin A selection method, α1,3Gal-negative cells are selected, regardless of whether inactivation of the second α1,3GT allele was caused by targeted disruption via the pPL680 vector or by any other mechanism. The fact that one normal-sized allele was observed (instead of two shorter knockout alleles) indicated that knockout of the second α1,3GT allele was due to mechanisms other than targeted homologous recombination-mediated disruption, promoter dysfunction, or mRNA missplicing and instability.

To identify the nature of the inactivation event for the second allele, we subcloned and sequenced α1,3GT cDNAs from all four cell lines (B1-1, B1-2, B1-3, and B1-4). Sequencing results revealed that there was a T-to-G transversion at the second base pair of exon 9 in the nontargeted α1,3GT allele of B1-1,

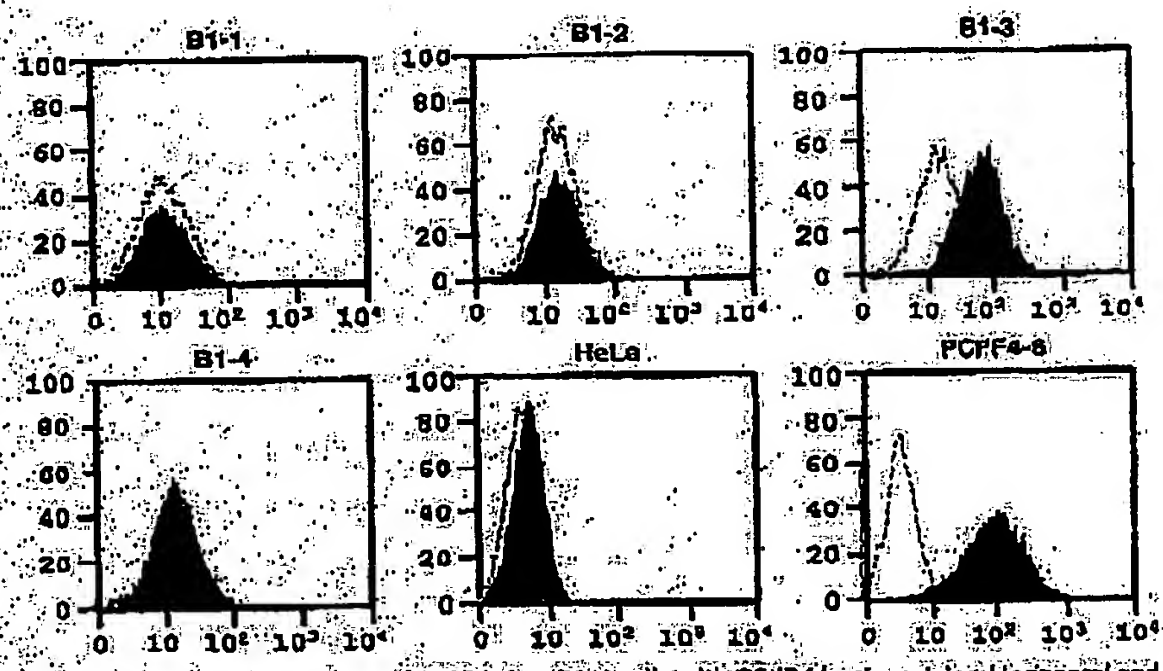


Fig. 1. Flow cytometry analysis of 680B1-1 to B1-4 cells with GS-IB4 lectin staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. Dotted line represents unstained cells analyzed by a fluorescence-activated cell sorter (Becton-Dickenson, Franklin, NJ). Shadow represents cells stained with fluorescein isothiocyanate–labeled GS-IB4 lectin (EY Laboratories, Inc., San Mateo, CA). B1-1, B1-2, B1-3, and B1-4 are fetal fibroblasts derived from four day-39 fetuses. HeLa cells, a human cell line, were used as the negative control and PCFF4-6 cells, which were the parent cells for heterozygous and DKO of the α1,3GT gene, were used as the positive control.

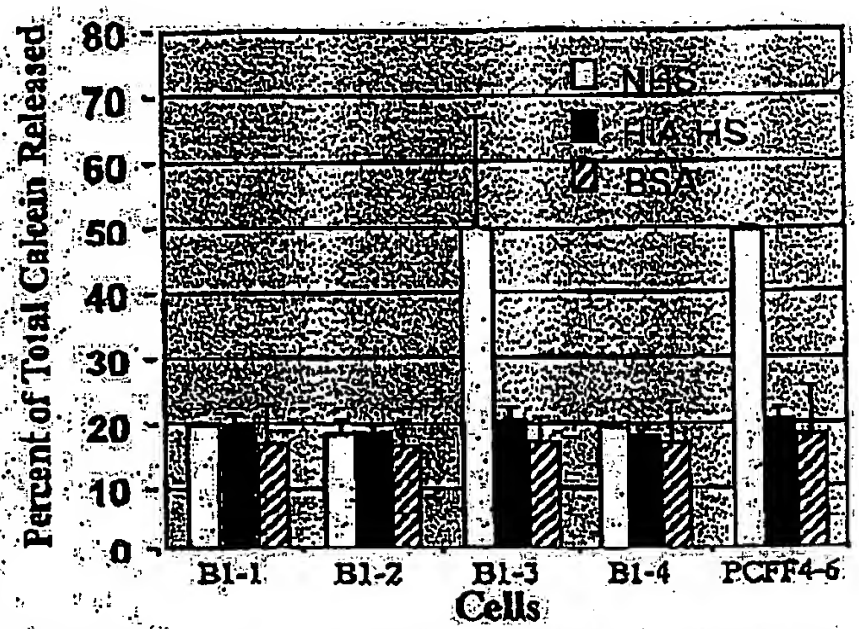


Fig. 2. Complement lysis assay for DKO fetal fibroblasts and wild-type pig cells. Results are the average of three individual assays. Open box represents NHS. Solid box represents heat-inactivated human serum (HIA-HS) as the negative control, and hatched box represents bovine serum albumin (BSA) as the reagent control. About 20% calcein release (no cells lysed) is the base value for the negative control (HIA-HS) and the reagent control (BSA). For quality control and reproducibility purposes, we did not use fresh human serum for the assay, which usually gives about 90% calcein release. About 50% calcein release (about 40% of cells lysed) from wild-type pig cells is typical with commercial serum (frozen and lyophilized) from Sigma.

B1-2, and B1-4 cells, but not in B1-3 cells or in the first knockout allele of all four cell lines. This T-to-G transversion in the α1,3GT coding region caused a single amino acid change from tyrosine to aspartic acid in the α1,3GT protein (Fig. 3). Although this mutation has not been observed in the inactivated α1,3GT gene of humans or higher primates (*14*), it is likely that the change of tyrosine, a hydrophobic amino acid, to aspartic acid, a hydrophilic amino acid, could disrupt α1,3GT function. Crystal structure analysis of bovine α1,3GT protein supports this speculation and shows that this tyrosine is at the center of the catalytic domain of bovine α1,3GT protein and is involved in uridine 5′-diphosphate–Gal binding (*15*, *16*).

To further confirm that the mutated cDNA cannot make functional α1,3GT protein, we cloned α1,3GT cDNAs from the nontargeted allele of B1-1 to B1-4 cells and wild-type pig cells into an expression vector and transfected them into human HeLa cells, which normally do not express α1,3GT protein. HeLa cells transfected with cDNA expression vectors from B1-1, B1-2, and B1-4 cells were negative for GS-IB4 lectin staining, indicating that the transfected pig cDNA from these cells did not make functional α1,3GT protein. In contrast, HeLa cells transfected with the cDNA from B1-3 cells and wild-type pig cells were positive with GS-IB4 staining. These results verified that the point mutation in cDNA from the second allele of the α1,3GT gene in B1-1, B1-2, and B1-4 cells resulted in synthesis of a defective α1,3GT protein. Although toxin A selection was repeated several times on 657A-III 1-6 cells, with or without pPL680 vector transfection, no additional toxin A–resistant colonies were detected.

We performed somatic cell nuclear transfer (cloning) with all three DKO cell lines as described in (*7*). We transferred cloned embryos into 16 estrus-synchronized recipient gilts. Ten initial pregnancies were established, only two of which went to term. Two pregnancies were lost before day 30, five were lost between day 30 and day 40, and one was lost by day 60. The first five female α1,3GT DKO piglets (761-1 to 761-5), cloned from 680B1-2 cells, were born on 25 July 2002. One piglet (761-1) died shortly after birth, and necropsy revealed an enlarged tongue and unusually large kidneys. We have observed this phenotype in a few other α1,3Gal-positive cloned pigs, and it appears to be a function of the cloning process (incomplete reprogramming) and not the α1,3GT gene knockout per se. The other four DKO piglets were of normal size and healthy. Aorta endothelial cells and muscle and tail fibroblasts isolated from the dead piglet (761-1) were negative with GS-IB4 lectin staining. FACS analysis of muscle fibroblasts from piglet 761-1 also showed a negative result for GS-IB4 binding. Neonatal tail fibroblasts isolated from the four healthy piglets, when analyzed by FACS with GS-IB4, were all negative (fig. S2). Tissue sections of liver, kidney, spleen, skin, intestine, muscle, brain, heart, pancreas, lung, aorta, tongue, umbilicus, and tail obtained from piglet 761-1 were all negative with GS-IB4 staining, indicating a complete lack of detectable cell surface α1,3Gal epitopes. The GS-IB4 staining results for liver sections from a newborn wild-type piglet and from piglet 761-1 are shown in fig. S3. Southern blot and sequencing analysis of DNA samples from all five piglets confirmed the targeted disruption of the first allele of the α1,3GT gene and the T-to-G point mutation in the second base of exon 9 in the second allele of the α1,3GT gene. It has been reported that α1,3GT DKO mice developed cataracts at 4 to 6 weeks of age (*17*). Physical examination of the four piglets at 7 weeks of age did not reveal any abnormalities or cataracts. We will continue to monitor the piglets for the presence of cataracts.

We performed an in vivo immunogenicity test with α1,3GT-knockout mice. We injected islet-like cell clusters (ICCs) isolated from the pancreas of piglet 761-1 intraperitoneally into α1,3GT knockout mice. We used ICCs from a neonatal wild-type piglet as a control. As shown in fig. S4, no increase in the titer of immunoglobulin M (IgM) to α1,3Gal was observed in α1,3GT knockout mice after injection with ICCs from the α1,3GT DKO piglet, in contrast to significant IgM titer increases observed in those mice injected with wild-type piglet ICCs. This result clearly demonstrates that the DKO piglet cells do not make any α1,3Gal epitopes.

Thus, we have successfully produced four α1,3GT-deficient piglets by a toxin A–mediated selection method. Although our intent was to knock out the second allele of the α1,3GT gene by homologous recombination, this did not occur. Instead, because we used this powerful selection method, which allows us to isolate any event that results in loss of α1,3GT activity, we discovered a mutation in the second allele of the α1,3GT gene. Had we used standard selection methods with puromycin or hygromycin, we would not have found the mutation. Although the rate of spontaneous mutation in the pig genome is very low [about $4 \times 10^{-8}$ for a spontaneous mutation per replication (*18*) in a mammalian gene similar in size to the α1,3GT gene], toxin A selection still enabled us to detect this crucial mutation. Clearly inactivation of the α1,3GT protein by this point mutation is a better outcome than by gene targeting with the pPL680 vector. It provides the opportunity to produce α1,3GT-deficient pigs without any antibiotic-resistance genes or other foreign DNA sequences, which should facilitate regulatory approval and, potentially, make a safer product for human use. It is certain that this point mutation will be maintained in the genome of these DKO pigs and their offspring, just as the few critical point mutations in the α1,3GT gene of humans and higher primates have been maintained over 20 million years (*14*). This genomic stability is not only due to the rarity of a reverse mutation event [about $5 \times 10^{-11}$ per replication (*18*) for mammals] but, more importantly, the strong selection pressure against α1,3Gal-positive cells by the presence of antibodies to α1,3Gal in α1,3Gal-negative animals. Our results have demonstrated that removal of α1,3Gal epitopes on pig cells did not preclude development in utero, even though pig cells express up to 500 times the number of α1,3Gal epitopes as do mouse cells (*4*, *19*). In addition, three consecutive rounds of cloning with rederived fetal cells did not appear to have a major detrimental effect on the overall development or health of the cloned pigs in this study. Analysis of tissues and organs from these α1,3GT DKO pigs in nonhuman primate models should provide clear indications of the involvement of α1,3Gal in HAR, AVR, and chronic rejection.

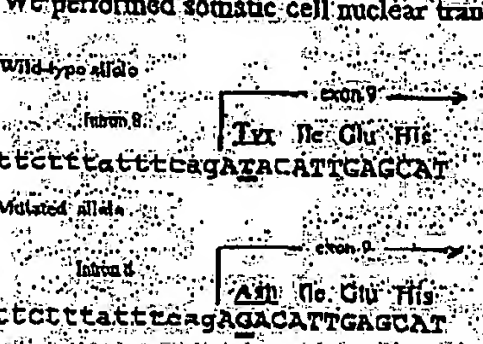


Fig. 3. Sequencing analysis of the α1,3GT gene from wild-type pig cells and DKO porcine fetal fibroblasts. Upper and lower alignment show nucleotide sequence of the α1,3GT intron 8–exon 9 boundary from wild-type pigs and the second allele of the DKO pig fetuses (B1-1, B1-2, and B1-4), respectively. Small letters and capital letters denote intron and exon sequences, respectively. Underlined capital letters indicate the nucleotide where the point mutation occurred. Amino acids deduced from the correspondent mutated and wild-type DNA sequence are underlined. No other mutations were found in the coding region of the α1,3GT gene from the second allele of the DKO pig fetuses in our genomic and reverse transcriptase–PCR libraries.

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12. pPL680 was made from three parts: a 1.8-kb PCR-

# Production of α-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations

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Edited by Rudolf Jaenisch, Massachusetts Institute of Technology, Cambridge, MA, and approved March 30, 2004 (received for review November 26, 2003)

**Hyperacute rejection of porcine organs by old world primate recipients is mediated through preformed antibodies against galactosyl-α-1,3-galactose (Galα-1,3-Gal) epitopes expressed on the pig cell surface. Previously, we generated inbred miniature swine with a null allele of the α-1,3-galactosyltransferase locus (*GGTA1*) by nuclear transfer (NT) with gene-targeted fibroblasts. To expedite the generation of *GGTA1* null pigs, we selected spontaneous null mutant cells from fibroblast cultures of heterozygous animals for use in another round of NT. An unexpectedly high rate of spontaneous loss of *GGTA1* function was observed, with the vast majority of null cells resulting from loss of the WT allele. Healthy piglets, hemizygous and homozygous for the gene-targeted allele, were produced by NT by using fibroblasts that had undergone deletional and crossover/gene conversion events, respectively. Aside from loss of Galα-1,3-Gal epitopes, there were no obvious phenotypic differences between these null piglets and WT piglets from the same inbred lines. In fact, congenital abnormalities observed in the heterozygous NT animals did not reappear in the serially produced null animals.**

Antibodies against galactosyl-α-1,3-galactose (Galα-1,3-Gal) residues on cell surface glycoproteins of pig cells mediate hyperacute rejection of porcine organs in primate model recipients and are the most immediate barrier to successful clinical xenotransplantation (1, 2). High levels of preformed "natural" antibodies against the Galα-1,3-Gal epitope are found in humans and old world primates, following evolutionary loss of the corresponding galactosyltransferase activity (encoded by *GGTA1*) (3). The presence of these antibodies, along with the high density of Galα-1,3-Gal residues on most pig cells (4), suggests that elimination of *GGTA1* function would provide a practical means of overcoming both hyperacute rejection and subsequent acute or chronic tissue damage associated with antibody binding to this epitope.

The lack of *GGTA1* function in humans and old world primates, along with the viability of *GGTA1* knockout mice produced with embryonic stem cell technology (5, 6), suggested that a knockout strategy might be biologically feasible in pigs. The cloning of sheep (7) and subsequently pigs (8–10) by nuclear transfer with somatic cells has made attempts to knockout the *GGTA1* locus in pigs technically feasible.

We have previously reported the generation of *GGTA1* heterozygous inbred miniature swine using nuclear transfer with gene-targeted fibroblasts (11). Starting with heterozygous fibroblasts from such animals, we now report the isolation of *GGTA1* null cells with spontaneous loss of the WT allele. The rate of loss of heterozygosity (LOH) was several orders of magnitude greater than typically expected, an observation that may be related to the inbred background of the heterozygous animals. LOH resulted in some cases from deletion of the WT allele and in others from either somatic crossing over or gene conversion. Similarly high rates of somatic recombination, subject to modulation by genetic background and chromosomal structure, have been reported in the mouse (12). Generation of healthy piglets with both hemizygous and homozygous *GGTA1* null cells demonstrates that such somatic LOH mutations can be introduced into large animal genomes by nuclear transfer, in a manner analogous to that using murine embryonic stem cell chimeras (13).

## Methods

***GGTA1* Heterozygous Cell Lines.** ***355-F1.*** Fetus 355-F1 was generated by nuclear transfer from cultured ear fibroblasts of pig O212-2, a *GGTA1* heterozygote in which one allele has been inactivated by homologous recombination with vector pGalGTΔS-Neo (11). Cells were isolated at day 33 of gestation by digestion with collagenase/thermolysin (Blendzyme 3, Roche Diagnostics, Indianapolis, IN) and cultured in Ham's Nutrient Mixture F10 (Invitrogen Life Technologies, Baltimore) containing 20% FBS.

***PL556.*** Piglet PL556 was derived by nuclear transfer. The donor cell clone, F501-F4, was produced by targeting of fetal fibroblasts from WT fetus F501 with vector pGalGTΔS-Neo, as described (11). PL556 cells were cultured in high glucose DMEM (Invitrogen Life Technologies, Baltimore) containing 10% FBS and 0.1 mM 2-mercaptoethanol.

Cell culture for all work reported here, beginning with tissue acquisition, was done in the absence of G418.

**Nuclear Transfer (NT).** For generation of piglets from clonal null cell lines, oocytes from sow ovaries were purchased (BoMed, Madison, WI), and NT was performed as described (11). The surviving embryos, possessing an intact plasma membrane, were selected for transfer into recipients after culture for 18–22 h. Potential domestic recipients were heat checked twice a day. Depending upon the exact time of estrus, 100–180 NT-derived embryos were transferred into recipient oviducts 5–17 h or 20–36 h after the onset of estrus for day 0 and day 1 recipients, respectively.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: NAb, natural antibody; LOH, loss of heterozygosity; NT, nuclear transfer; Galα-1,3-Gal, galactosyl-α-1,3-galactose; LDH, lactate dehydrogenase; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt].

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‡D.K.-S., M.D., S.A., J.L.G., D.J.J.R., D.H.S., and R.J.H. are or have been employed by Immerge Biotherapeutics, and S.R.W., M.L.A., J.B., G.D.M., and E.J.F are or have been employed by Infigen, Inc., with a continuing interest in xenotransplantation. These authors have declared a potential conflict of interest solely for this reason.

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For NT with nonclonal null cell-enriched populations, oocyte maturation, NT, embryo culture, and embryo transfer into recipient females were performed as described (8), except that female recipients were selected that exhibited first standing estrus within 12 h of cybrid activation.

**Baboon Natural Antibody.** Anti-Gal$\alpha$-1,3-Gal antibodies from naive baboon plasma (natural antibody, NAb) were affinity-purified by absorption to Gal$\alpha$-1,3-Gal LB-VI matrix columns (14) (Alberta Research Council, Alberta, Canada). The bound NAb was eluted from the column in 0.25% acetic acid, neutralized, and dialyzed against PBS before concentration and sterile filtration.

**Null Cell Selection.** To enrich for *GGTA1* null cells in fibroblast cultures, 355-F1 cells and PL556 cells were cultured in F10 medium containing 20% FBS and 20 $\mu$g/ml gentamycin on collagen I-coated dishes at 5% $CO_2$, 3% $O_2$, and 37°C. The above cell lines were treated in suspension at $2 \times 10^6$ cells/ml in 100 $\mu$g/ml affinity-purified baboon NAb in media for 30 min at room temperature with mixing. After washing, cells were then treated with 12.5% baby rabbit complement (Pel-Freez Biologicals) containing DNase I (10 $\mu$g/ml) in media for 45 min at room temperature with mixing. Surviving cells were counted and plated in bulk culture and expanded for subsequent treatments. This selection was repeated three times for 355-F1 cells and twice for PL556 cells. Selections were performed every 7–10 days, with the fourth selection of 355-F1 performed 3 days after the third selection. Before each NAb/complement selection, cells were analyzed for the presence of Gal$\alpha$-1,3-Gal epitopes with FITC-conjugated BS-I-$B_4$.

For clonal selection, 355-F1 cells were treated twice in suspension as above with 50 $\mu$g/ml NAb and 12.5% complement, with 4 days between treatments. After the second treatment, cells were plated at 5 and 10 cells/well in collagen I coated 96-well plates. *In situ* treatments with 100–500 $\mu$g/ml NAb for 1 h at 37°C and 12.5% complement for 1 h at 37°C were performed every other day for treatments 3–5. Wells containing patches of cells covering >15% of the well were transferred to a 48-well plate and treated the following day *in situ* with 500 $\mu$g/ml NAb and complement. Cells were passaged for molecular analysis, BS-I-$B_4$ analysis, and freezing.

**Flow Cytometry Analysis.** Gal$\alpha$-1,3-Gal epitope expression was analyzed with FITC-conjugated BS-I-$B_4$ lectin (Sigma). Unfixed cells were stained for 5 min at 37°C in 4 $\mu$g/ml lectin, washed, and then resuspended in buffer containing propidium iodide (PI). Fluorescence data were collected on a Becton Dickinson FACScan, and analysis of PI excluding cells was performed by using CELLQUEST flow cytometry software (BD Immunocytometry Systems).

**Quantitative Southern Blots.** Genomic DNA was digested with *Afl*III, which generates a 1,280-bp fragment of the WT *GGTA1* allele (sites at bp 9 and bp 1,289 of GenBank accession number AF221517) and a 2,330-bp fragment of the pGalGTΔS-neo targeted allele (11). Southern blots were simultaneously probed with $^{32}$P-labeled RNA transcripts from the exon 9 portion of this fragment (bp 776–891 of GenBank accession number AF221517) and a portion of the porcine DQ-$\beta$ locus (bp 901-1015 of GenBank accession number M31497). Phosphorscreen autoradiography was performed on a STORM 820 Optical Scanner, and area quantitation was done with IMAGEQUANT 5.2 software (Molecular Dynamics).

**Microsatellite Analysis.** PCR was performed by using WellRED-labeled primers, and the reactions were analyzed on a CEQ2000 sequence analyzer (Beckman Coulter). Markers Sw2518 and Sw1430 map to porcine chromosome 1 at ≈67 cM and 58 cM, respectively; combined radiation hybrid and genetic data place the *GGTA1* locus at ≈115–122 cM (www.genome.iastate.edu/pig). Heterozygosity of marker Sw2518 in fetus 355-F1 and Sw1430 in piglet PL556 was confirmed by segregation of alleles within the respective inbred miniature swine lineages.

**Complement-Mediated Lysis.** Lysis [lactate dehydrogenase (LDH) release] and metabolism {conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]} were measured by using the Cytotox 96 NonRadioactive Cytotoxicity and CellTiter 96 Aqueous One Solution Cell Proliferation assays (Promega), respectively. Human serum was prepared by heat inactivation of a pool from 10 untyped individuals. Preparation of affinity-purified baboon NAb was as described above. Baby rabbit complement was obtained from Pel-Freez Biologicals. Normal human dermal fibroblasts (Cambrex Bioscience, Walkersville, MD) served as Gal$\alpha$-1,3-Gal negative cell controls.

Two days after plating in triplicate wells, subconfluent fibroblasts were incubated in medium (Ham's Nutrient Mixture F10, 5% FBS) containing NAb or human serum for 30 min at 37°C. The cells were washed twice with Ham's Nutrient Mixture F10 and then incubated for 60 min at 37°C in the above medium containing 12.5% rabbit complement. Medium from this incubation was assayed for LDH release. Remaining cells were incubated in medium containing 16.5% MTS for 2.5–3 h. After incubation, 25 $\mu$l of 10% SDS was added to all wells, and the medium was assayed for MTS conversion. Samples from triplicate "no cell" controls for each treatment condition were used to correct for assay background. For MTS assays, corrected average values are expressed as the percentage of corrected absorbance without NAb or serum for each line. For LDH assays, corrected average values are expressed as the percentage of corrected average absorbance after detergent lysis for each line. LDH release using anti-pig pan tissue mAb 1030h-1-19 (BD Biosciences PharMingen) was used as a positive control for porcine cell lysis, with similar titrations obtained for all three porcine fibroblast lines (data not shown).

## Results

**Selection of *GGTA1* Null Lines and Clones from Fetal and Neonatal Heterozygous Cell Lines.** Two sources of heterozygously targeted primary fibroblasts were chosen for selection of *GGTA1* null cells (Fig. 1). Fibroblasts from fetus 355-F1 were isolated at 33 days gestation after NT using ear fibroblasts from *GGTA1* heterozygous gilt O212-2. O212-2 was itself generated by NT by using gene-targeted fetal fibroblast clone F7-H6 as described (11). Similarly, ear fibroblasts from male *GGTA1* heterozygous neonate PL556 were isolated after NT by using gene-targeted fibroblast clone F501-F4.

Approximately $1.5 \times 10^7$ cells from established cultures of both sources were depleted of Gal$\alpha$-1,3-Gal epitope-bearing cells by lysis with affinity-purified baboon antibodies against the epitope in combination with complement. Enrichment for Gal$\alpha$-1,3-Gal-negative cells was monitored by flow cytometry analysis with FITC-labeled BS-I-$B_4$ lectin, which binds specifically to Gal$\alpha$-1,3-Gal epitopes. Initial depletions resulted in recovery of ≈0.1% of the cells, 7–15% of which were Gal$\alpha$-1,3-Gal negative. After 3–4 rounds of selection and expansion, these populations were essentially devoid of BS-I-$B_4$ binding cells (Fig. 2). DNA prepared from the null selected populations was analyzed by PCR that distinguishes WT and targeted *GGTA1* alleles. The absence of a readily detectable WT band indicated that the vast majority of these cells had undergone at least partial loss of the WT allele (Fig. 2). This loss of heterozygosity in the *GGTA1* null cells is compatible with chromosome loss and reduplication, interstitial deletion, or somatic recombination.

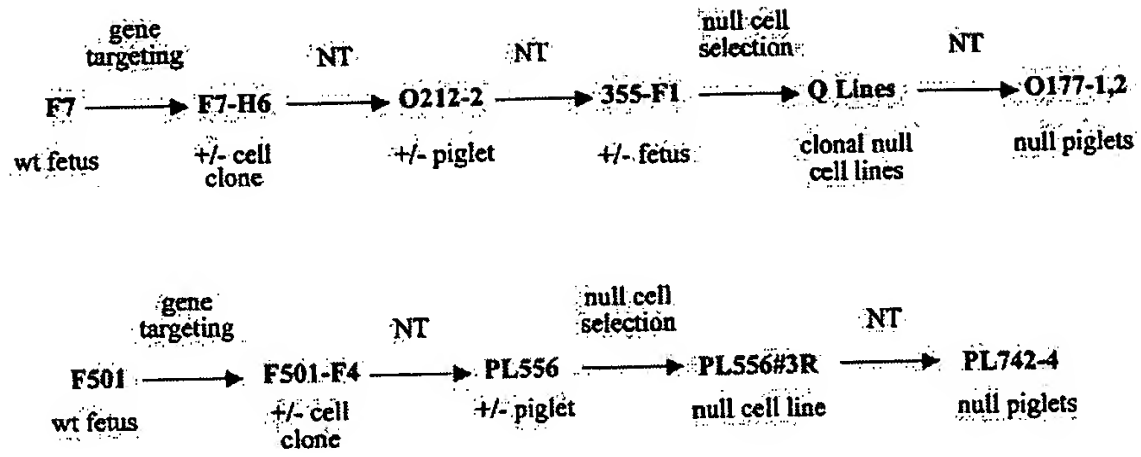


**Fig. 1.** Derivation of *GGTA1* null pigs.

Clonal null lines were isolated from a 355-F1 cell population that had been previously enriched for null cells by NAb/complement depletion. The recovery rate of null clones from the initial heterozygous lines was $\approx 10^{-4}$ in two separate trials. Microsatellite analysis of 28 clones with centromere proximal marker Sw2518 (heterozygous in 355-F1) revealed that all clones remained heterozygous at this locus, indicating that chromosome loss and reduplication was not the mechanism for loss of heterozygosity (LOH) in these clones. DNA samples from four of these clonal lines (Q2, Q9, Q32, and Q37), along with the 355-F1 and PL556 null selected populations, were analyzed by quantitative Southern blotting by using an exon 9 *GGTA1* probe present in both the targeted and WT alleles. A probe for the nonlinked porcine SLA *DQβ* gene served as a diploid copy number control (Fig. 3). In both null selected populations and all four clones, only a targeted length *GGTA1* allele was detected. In two null clones (Q2 and Q32) a *DQβ/GGTA1* signal ratio of $\approx$2:1 was obtained, demonstrating deletion of at least a portion of the WT *GGTA1* allele present in 355-F1. In comparison, clones Q9 and Q37 had *DQB/GGTA1* signal ratios of $\approx$1:1, indicating that loss of heterozygosity in these clones occurred through either somatic crossing over or gene conversion. Efforts to distinguish between these mechanisms by microsatellite analysis were unsuccessful because no heterozygous markers distal to Sw2185 were identified in fetus 355-F1 from among 24 tested. Sequencing of the junctions between exon 9 and the G418 selection cassette in clones Q9 and Q32 revealed no evidence of nucleotide heterozygosities (data not shown).

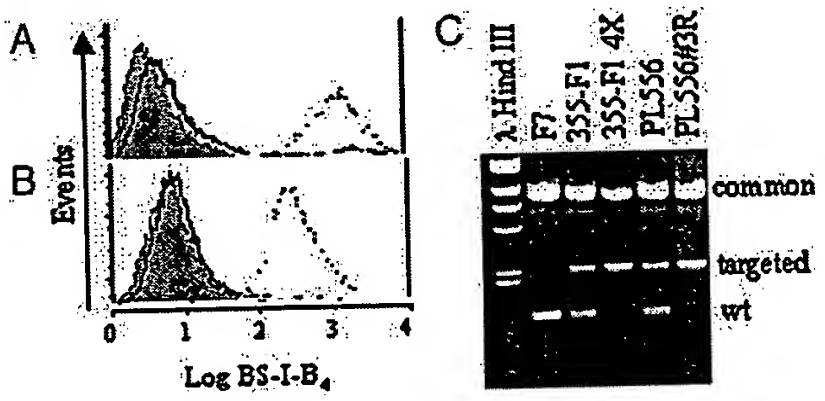


**Fig. 2.** Selection of *GGTA1* null cells from heterozygously targeted fibroblasts. Fibroblasts from heterozygous fetus 355-F1 (*A*) and neonatal piglet PL556 (*B*) were analyzed by flow cytometry for binding to FITC-conjugated Gal$\alpha$-1,3-Gal-specific lectin BS-I-$B_4$. 355-F1 4X and PL556#3R populations (solid lines) were selected four and three times, respectively, by lysis with affinity-purified baboon NAb and complement. Stained cells before selection (broken lines) and unstained selected populations (solid lines) served as positive and negative controls for BS-I-$B_4$ binding. (*C*) Genomic DNA from the above cell populations (and WT fetus F7) was analyzed by PCR by using a forward primer upstream of the selection cassette of the *GGTA1* targeting vector (F527) and a reverse primer (GR2520) downstream of the vector end, as described (11). *Sac*I digestion yields a 2,300-bp band from the targeted GGTA1 allele, a 1,250-bp band from the WT allele, and a 7,900-bp band common to both alleles. The WT band is not detected after NAb/complement selection.

**Generation of *GGTA1* Null Pigs by NT.** NT was performed by using the four clonal fetal cell lines characterized by Southern analysis (Q2, Q9, Q32, and Q37), as well as the null cell population selected from neonate PL556. Embryo transfer results are summarized in Table 1.

Transfers with embryos reconstructed using the 355-F1-derived clonal lines Q2 and Q32 each resulted in one pregnancy to term. A single mummy was recovered from the recipient of Q2-derived embryos. The recipient carrying Q32-derived embryos delivered two live born female piglets, O177-1 and O177-2,

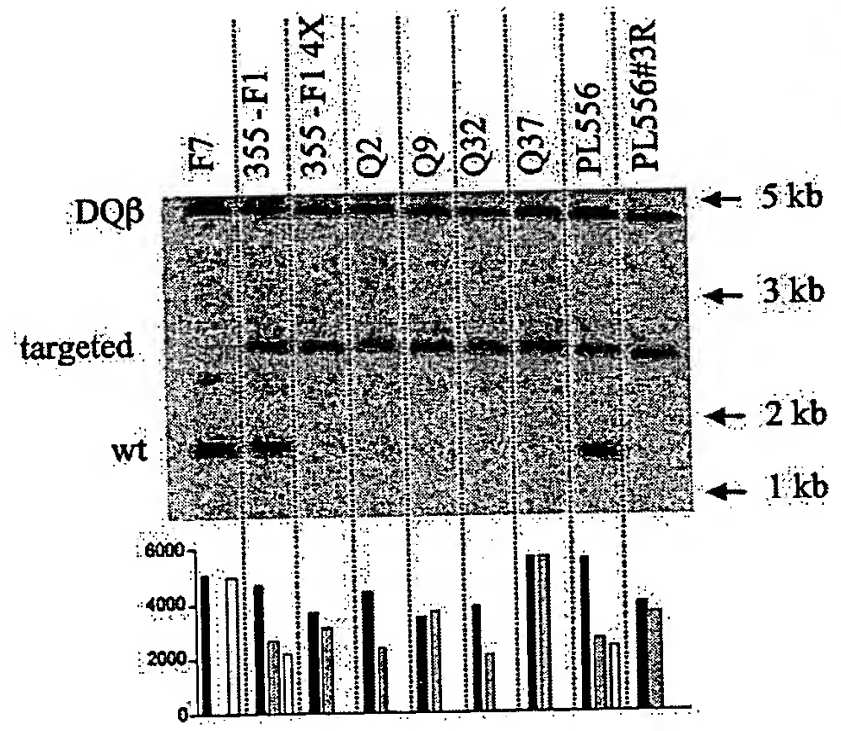


**Fig. 3.** Quantitative Southern blot analysis of NT donor lines. Genomic DNA from the indicated sources was digested with restriction enzyme *Afl*III, Southern blotted, and hybridized simultaneously with a 116-bp probe from exon 9 of the *GGTA1* locus and a 107-bp probe from the porcine SLA *DQβ* locus. The *GGTA1* probe hybridizes a 1.3-kb WT fragment and a 2.3-kb gene-targeted fragment containing an IRES-neo selection cassette. DNA from WT (F7) and heterozygous (355-F1 and PL556) fibroblasts, before NAb/complement selection, served as controls. 355-F1 4X and PL556#3R samples were prepared from cell populations selected four and three times, respectively, with affinity-purified baboon NAb and complement. Q series samples were from clonal cell lines isolated from 355-F1 fetal fibroblasts. Signal quantitation was performed on a Storm 820 PhosphorImager and graphed as absolute values for the *DQβ* locus (■), targeted *GGTA1* allele (▣), and WT *GGTA1* allele (□).

**Table 1. Nuclear transfer with *GGTA1* null cells**

| Cell line | Transfers | Pregnant | Births |
|---|---|---|---|
| Q2 | 4 | 2 | 1 |
| Q9 | 3 | 2 | 0 |
| Q32 | 6 | 3 | 1 O177-1 and -2 born 11/18/02 |
| Q37 | 5 | 3 | 0 |
| PL556#3R | 30 | 7 | 2 PL742–744 born 1/13/03 |

by means of caesarian section. O177-1 weighed 575 g at birth, was healthy, and continued normal growth thereafter. Littermate O177-2 was undersized (275 g) and died shortly after delivery.

Transfers with embryos reconstructed using the null selected cell population from neonate PL556 also resulted in two pregnancies to term. Two dead, late-stage fetuses were obtained by caesarian section from one surrogate. The other surrogate farrowed three healthy male piglets (PL742, PL743, and PL744) weighing 550, 320, and 450 g, respectively.

No evidence of cataract formation, seen previously in *GGTA1* knockout mice (5, 6), or other phenotypic differences between the *GGTA1* null pigs and naturally produced WT miniature swine were observed.

**Molecular Analysis of *GGTA1* Null Pigs.** DNA from the five NT piglets described above was analyzed by quantitative genomic Southern blotting and microsatellite analysis. Piglets O177-1 and O177-2 were found to be hemizygous for the targeted *GGTA1* allele, consistent with their derivation from the Q32 donor cell clone (Fig. 4). Also as expected, both piglets were heterozygous for marker Sw2518.

In contrast to the Q32 derived piglets, all three piglets (PL742–744) derived from the nonclonal PL556 null selected cell line were homozygous for the gene-targeted *GGTA1* allele (Fig. 4). A single heterozygous microsatellite marker, Sw1430, was found from among 26 markers tested in piglet PL556. This centromere proximal marker remained heterozygous in all three

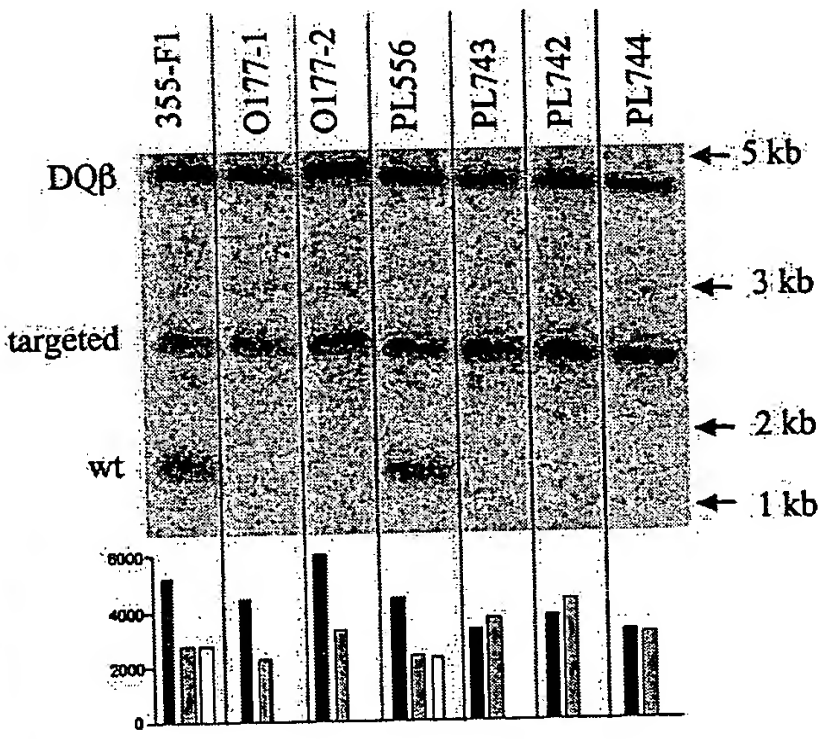


**Fig. 4.** Quantitative Southern blot analysis of *GGTA1* null piglets. DNA from piglets O177-1 and O177-2 (produced by NT with null fibroblast clone Q32) and piglets PL742–744 (produced by NT with the PL556#3R NAb/complement selected fibroblast population) was analyzed as described in the legend to Fig. 3. DNA from heterozygous 355-F1 and PL556 fibroblasts, without NAb/complement selection, served as controls. Shown are *DQβ* locus (■), targeted *GGTA1* allele (▩), and WT *GGTA1* allele (□).

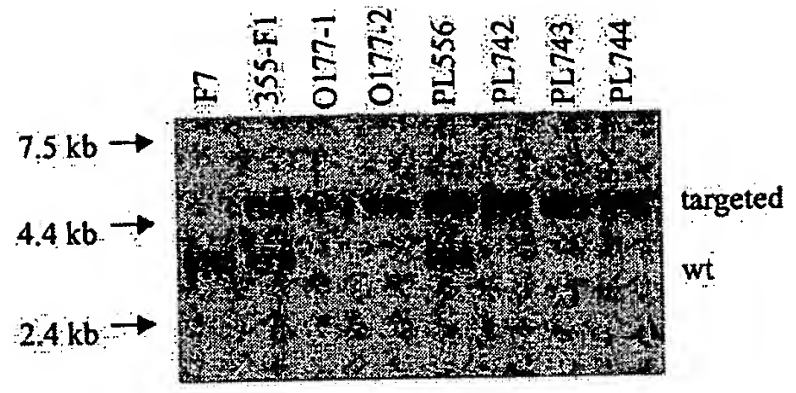


**Fig. 5.** *GGTA1* expression in fibroblasts from null piglets and progenitors. A Northern blot of poly(A)+ RNA was hybridized to a 1.4-kb probe containing portions of exons 2–9 of the *GGTA1* gene. WT F7 fibroblasts express a 3.6-kb transcript whereas cells from 355-F1 and PL556 heterozygotes express both a 3.6-kb transcript from the WT locus and a 4.7-kb transcript from the targeted locus. Only the 4.7-kb transcript is detected in fibroblasts from the null piglets.

PL556 derived *GGTA1* homozygous piglets. As with homozygous cell clones Q9 and Q37, no nucleotide heterozygosities were found at the junctions of *GGTA1* exon 9 and the G418 selection cassette in the three homozygous piglets (data not shown). Consistent with the hemizygous and homozygous targeted genotypes of these piglets, only RNA compatible with transcription from a targeted locus was observed upon Northern blot analysis (Fig. 5).

**Phenotypic Characterization of Cells from *GGTA1* Null Piglets.** Fibroblast cultures from ear explants of the four surviving null piglets were stained with FITC-labeled BS-I-$B_4$ lectin and examined by flow cytometry for evidence of cell surface Galα-1,3-Gal epitopes (Fig. 6 *A* and *B*). No fluorescence above that obtained

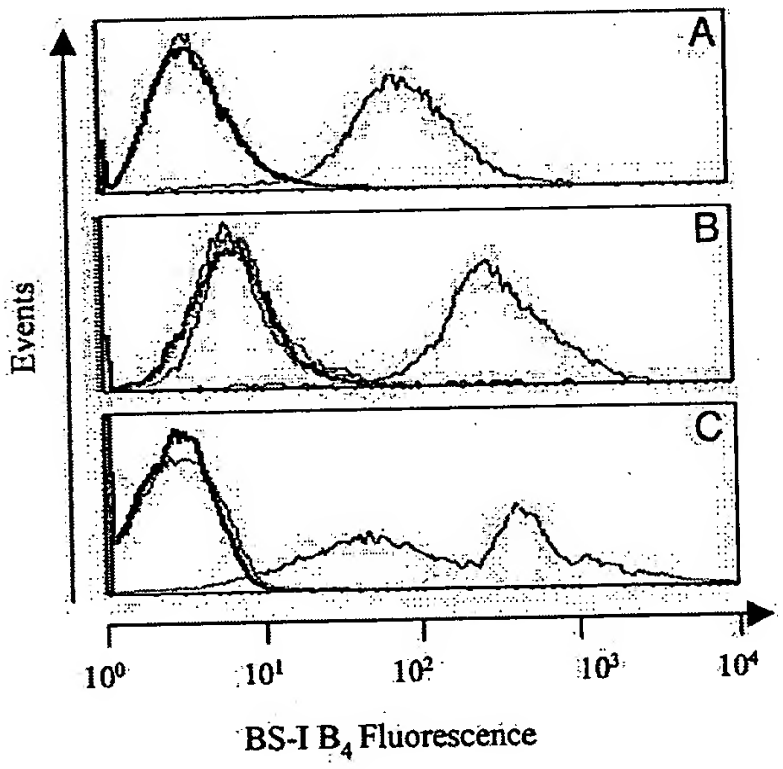


**Fig. 6.** Flow cytometry analysis of Galα-1,3-Gal epitopes on *GGTA1* null piglets and progenitors using BS-I-$B_4$ lectin. Stained heterozygous or WT cells (red) and unstained cells (black) served as positive and negative controls. (*A*) Stained (blue) and unstained (black) ear fibroblasts from *GGTA1* null piglet O177-1 and fetal fibroblasts from heterozygous progenitor 355-F1 (red). (*B*) Stained ear fibroblasts from null piglets PL742 (orange), PL743 (blue), and PL744 (green); stained (red) and unstained (black) fetal fibroblasts from heterozygous progenitor 355-F1. (*C*) Stained (blue) and unstained (black) multilineage white blood cells from O177-1 at 6 weeks of age and stained WBC from an age-matched WT control (red).

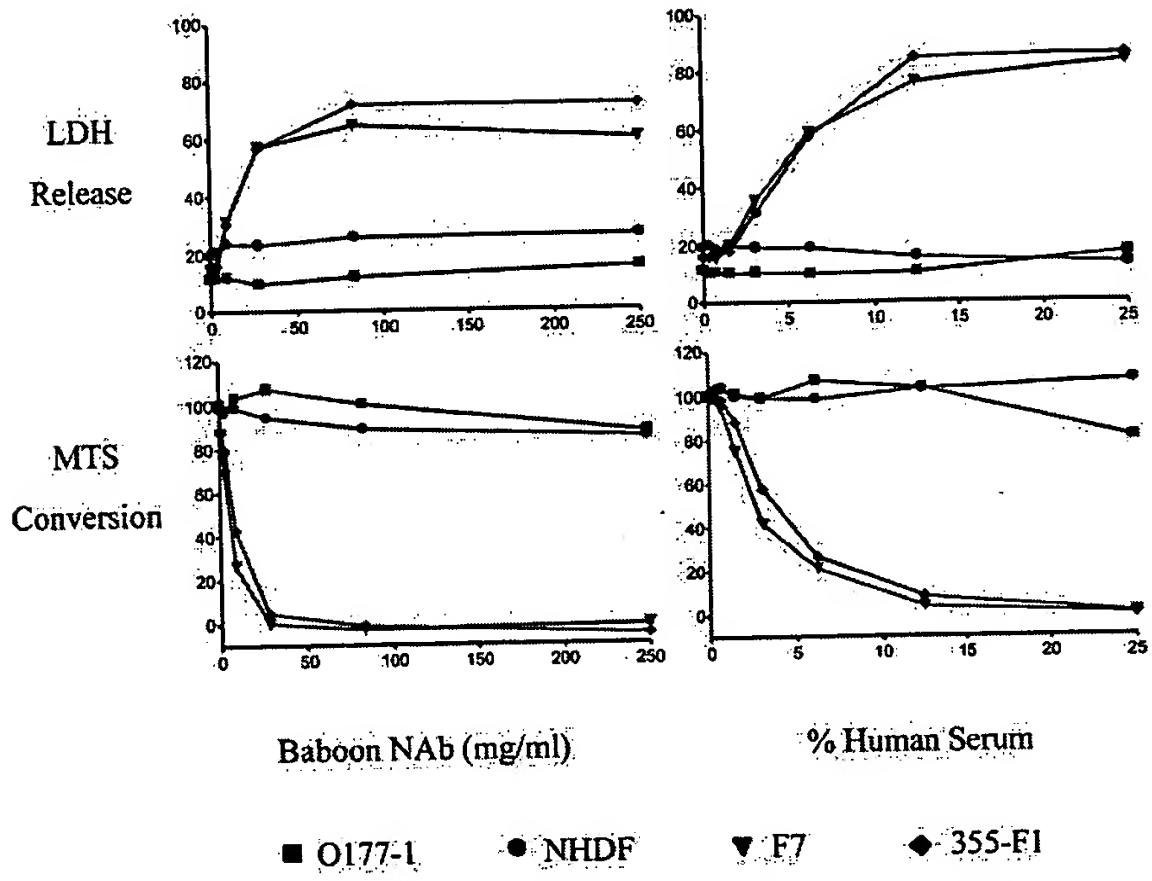


**Fig. 7.** Complement-mediated lysis of cells after incubation with purified baboon NAb or human sera. Fibroblasts from *GGTA1* null piglet O177-1, WT progenitor fetus F7, and heterozygous progenitor fetus 355-F1 were incubated with the indicated concentrations of affinity-purified polyclonal baboon NAb or heat-inactivated pooled human sera before lysis with rabbit complement. Normal human dermal fibroblasts (NHDF) served as a Galα-1,3-Gal negative control. Release of LDH is expressed as percent of total activity after detergent lysis. Residual metabolic activity, measure as MTS conversion, is expressed as percent of conversion without incubation in complement. Data are the average of three trials.

with unstained fibroblasts was observed. Similar results were obtained with Galα-1,3-Gal-specific mAb M86 (not shown). Epitope expression was also examined on multilineage white blood cells from O177-1 at 6 weeks of age, with none detectable (Fig. 6*C*).

Susceptibility of fibroblasts from null piglet O177-1 to complement-mediated lysis by purified baboon anti-Galα-1,3-Gal antibodies and heat inactivated human sera was assessed by using enzyme release and metabolic activity assays (Fig. 7). With purified antibodies, the $EC_{50}$ for WT and *GGTA1* heterozygous fibroblasts was <20 μg/ml in both assays whereas concentrations up to 250 μg/ml had no effect on O177-1 cells. Similarly, O177-1 fibroblasts also have far greater resistance to human sera and complement although partial lysis and metabolic inhibition were seen at the highest serum concentration.

## Discussion

Using NT with cells selected for loss of GGTA1 expression from gene-targeted heterozygous cell populations, we have produced healthy null piglets with two distinct LOH genotypes. The stringent selection for loss of function available for this locus permitted efficient selection of *GGTA1* null cell clones from heterozygous fetal cell cultures and sufficient enrichment from heterozygous neonatal cell cultures for use directly in NT.

The WT fetal progenitors for the piglets reported here were two inbred miniature swine, with inbreeding coefficients of 0.86 and 0.91 for fetuses F7 and F501, respectively. Mouse cloning experiments have demonstrated a severe decrease in viability of highly inbred embryos generated entirely by NT and, from many strains, an absolute failure to obtain viable mice (15, 16). Inbreeding in the miniature swine lines, at least to this point, has not resulted in a dramatic decrease in viability although some decrease in NT efficiency in comparison with commercial lines used in unrelated studies seems likely. However, the nature and frequency of spontaneous second allele mutations seems to be greatly influenced by the inbred genetic background. The recovery rate of mutant cells we observe resulting from LOH, $\approx 10^{-4}$, is several orders of magnitude greater than that typically

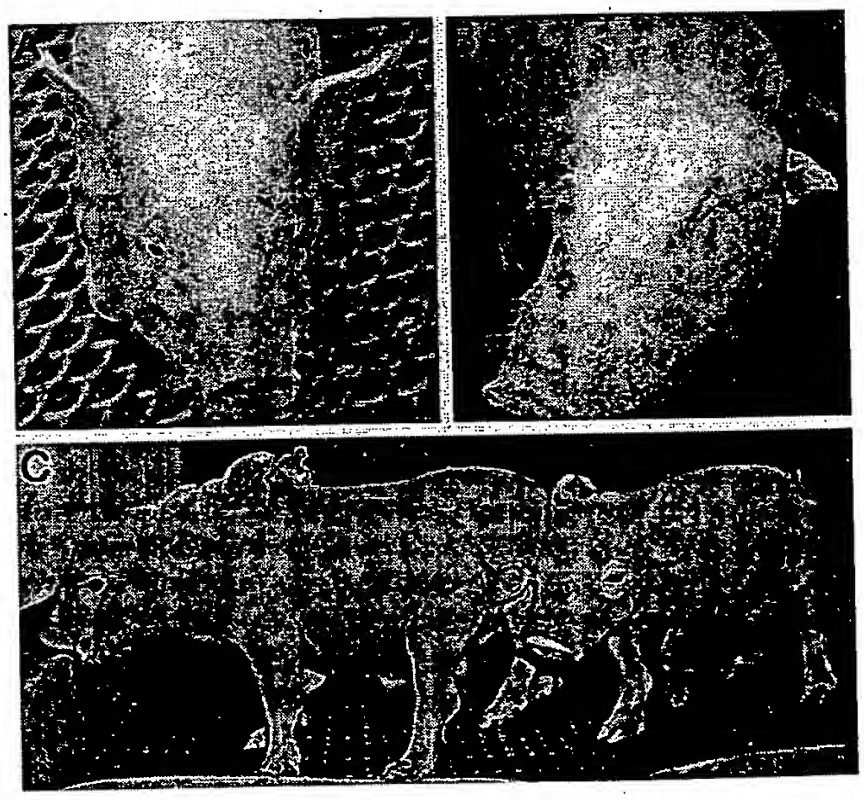

**Fig. 8.** *GGTA1* null and heterozygous pigs produced by NT. (*A*) Third-round NT piglet O177-1, produced using *GGTA1* null donor cells selected from second-round heterozygous fetus 355-F1 (age 66 days). (*B*) First-round NT pig O212-2 (11), ear fibroblasts from which served as NT donor cells for fetus 355-F1 (age 3 months). Eye and ear defects in this pig are not observed in O177-1, nor were they apparent at 33 days gestation in fetus 355-F1 or any of its 11 clonal littermates. (*C*) Second-round NT piglets PL742–744, produced using *GGTA1* null donor cells selected from first round heterozygous neonate PL556 (age 9 days).

expected in mammalian somatic cells. We have obtained similar results after null selection of fibroblasts from inbred *GGTA1* heterozygous piglets produced by mating (D.K-S. and D.J.J.R., unpublished observations), indicating that the high LOH rate is unrelated to the NT process itself. Furthermore, our results contrast markedly with those obtained in attempts to isolate *GGTA1* null cells on a commercial genetic background, in which mutants were obtained at rates of $<10^{-6}$. Phelps *et al.* (17) used Toxin A from *Clostridium difficile* to select against Gal$\alpha$-1,3-Gal epitope-bearing cells after transfection of heterozygously targeted cells with a second targeting vector. Although no doubly targeted cell clones were obtained, a single clone with a missense mutation in the nontargeted allele was isolated. Sharma *et al.* (18), using a similar antibody and complement selection with heterozygously targeted cells, isolated 11 resistant cell clones from a starting population totaling $2 \times 10^7$ cells. Southern blot analysis of two of these cell clones indicated loss of the nontargeted allele although the mechanism of loss was not further investigated and no pigs were produced by using the antibody resistant lines.

Although not observed in noninbred pigs, the rate of LOH in our inbred pig lines is almost identical to that reported for 2,6-diaminopurine (DAP) selection of spontaneously generated null fibroblasts from heterozygous mice bearing a gene-targeted *Aprt* allele. Shao *et al.* (19), using 129/Sv × C3H/HeJ hybrids, observed LOH in 92 of 113 DAP-resistant clones. In all cases, LOH resulted from somatic crossovers that occurred at various points, with a distribution biased toward the region just proximal to the *Aprt* locus. The propensity for somatic crossing over in this system was subsequently found to be dependent on chromosomal homology because hybrid mice bearing the relevant homologs from distantly related strains yielded much lower frequencies of spontaneous *Aprt* mutant fibroblasts and none of the mutant cell clones recovered were recombination derived (20). Thus, it seems likely that the *GGTA1* homozygous cells used in NT to produce the PL742–44 null piglets also arose through somatic crossing over. However, due to the lack of heterozygous markers in the F501 fetal progenitor, a gene conversion mechanism cannot be formally excluded. It is interesting to note that, if the homozygous donor cells did arise through a somatic crossover, then the pigs would carry a partial uniparental disomy for chromosome 1 distal to the crossover.

In slight contrast to the above *Aprt* studies, Ponomareva *et al.*, using the same gene-targeted *Aprt* allele on a C57BL/6 × DBA/2 background, selected spontaneously generated mutant cell clones with similarly high rates of both somatic crossover events and interstitial deletions (21). We have also recovered both hemizygous and homozygous null lines in all clonal null selection experiments performed with *GGTA1* heterozygous lines. Whereas neither deletional breakpoint seems to map within the *GGTA1* locus in hemizygous piglet O177-1, interstitial deletion mutant cell clones with one or both breakpoints within the *GGTA1* locus have been isolated in selections of other heterozygous fetal and neonatal ear fibroblast lines (D.K-S. and D.J.J.R., unpublished observations). Thus, for deletional events at least, LOH in our inbred derived fibroblast lines is a heterogeneous process.

Heterozygous piglets O212-2 and PL556, from which ear fibroblasts were isolated for second allele mutation selection, were produced early in the program and both were developmentally deficient. PL556 was undersized and died shortly after birth from acute respiratory distress. Although in good health and reproductively sound to date, O212-2 was born with one eye, small earflaps, and no patent ear canals (11). A variety of congenital abnormalities have been reported in cloned pigs (11, 22) as well as other species (22, 23), some of which arise through epigenetic errors in reprogramming (24). Unlike the joint and cardiopulmonary defects not uncommonly observed, the deficits in O212-2 must have arisen at a relatively early stage of development. Whereas it might be assumed that these aberrant phenotypes would be magnified only by additional *in vitro* manipulation and NT, the generation of normal, healthy piglets reported here (Fig. 8) clearly demonstrates that production of normal NT-derived progenitor animals is neither required, nor necessarily advantageous, when performing sequential genetic modifications.

Serial NT has allowed us to produce $\alpha$-1,3-galactosyltransferase null piglets in a considerably shorter timeframe than would be required for standard breeding from heterozygotes. Although the inbred miniature swine lines used here were chosen specifically for their advantages for xenotransplantation (25), they also serve to demonstrate the dramatic effect that genetic background can have on rates of spontaneous somatic mutational events in a large animal model. That somatic recombinational and deletional events are not necessarily associated with other deleterious events suggests that appropriate strain selection or construction may be useful in introducing some genetic modifications by means of NT.

We thank K. Barnhart, A. Bonk, T. Bruss, M. Duggan, J. Endres, P. Golueke, R. Koppang, G. Lange, K. Mallon, C. Patience, M. Samuel, and H. Schuurman for their contributions to this work. This work was supported in part by grants from the Advanced Technology Program (National Institute of Standards and Technology) and the Small Business Innovative Research Program (National Center for Research Resources, National Institutes of Health).

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*Transgenic Research* 10: 577–582, 2001.



*Short Communication*

# Production of transgenic miniature pigs by pronuclear microinjection

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Received 31 January 2001; revised 22 May 2001; accepted 29 May 2001

Key words: gene transfer, microinjection, miniature pig, transgenic

## Abstract

Miniature pig is an attractive animal for a wide range of research fields, such as medicine and pharmacology, because of its small size, the possibility of breeding it under minimum environmental controls and the physiology that is potentially similar to that of human. Although transgenic technology is useful for the analysis of gene function and for the development of model animals for various diseases, there have not yet been any reports on producing transgenic miniature pig. This study is the first successful report concerning the production of transgenic miniature pig by pronuclear microinjection. The huntingtin gene cloned from miniature pig, which is a homologue of candidate gene for Huntington's disease, connected with rat neuron-specific enolase promoter region, was injected into a pronucleus of fertilized eggs with micromanipulator. The eggs were transferred into the oviduct of recipient miniature pigs, whose estrus cycles were previously synchronized with a progesterone analogue. A total of 402 injected eggs from 171 donors were transferred to 23 synchronized recipients. Sixteen of them maintained pregnancy and delivered 65 young, and one resulted in abortion. Five of the 68 offspring (three of which were aborted) were determined to have transgene by PCR and Southern analysis. The overall rate of transgenic production was 1.24% (transgenic/injected eggs). This study provides the first success and useful information regarding production of transgenic miniature pig for biomedical research.

## Introduction

*Sus scrofa domestica*, including domestic pig and miniature pig, is attractive as a laboratory animal in a wide range of medical and pharmacological research field. Because of having similar anatomical and physiological characteristics to human (Bustad & McClellan, 1966; Douglas, 1972; Swindle et al., 1994). Domestic pig has been used for drug testing and a disease model (Mount & Ingram, 1971; Swindle & Smith, 1998). However, the body weight of domestic pig is over 150 kg at 4 months of age. In contrast, the body weight of Göttingen miniature pig is about 35–40 kg at 2 year-old (Bollen & Ellegaard, 1997), and that makes an easy handling and a low cost for maintaining the animals (Mount & Ingram, 1971; Bollen & Ellegaard, 1997). Therefore, the use of miniature pig is expecting in many biomedical fields (Svendsen, 1998).

Transgenic technology in domestic pig has already been recognized, and recently has received attention as organ donors in xenotransplantation (Cozzi & White, 1995) and also as a possible bioreactor producing therapeutic proteins (Cameron et al., 1994; Janne et al., 1994; Wagner et al., 1995; Wei, 1997). In view of the many advantages of miniature pig over domestic

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MOLECULAR REPRODUCTION AND DEVELOPMENT 60:189–195 (2001)

# Cloned Pigs Generated From Cultured Skin Fibroblasts Derived From a H-Transferase Transgenic Boar

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**ABSTRACT** Cloned pigs were produced from cultured skin fibroblasts derived from a H-transferase transgenic boar. One 90 day fetus and two healthy piglets resulted from nuclear transfer by fusion of cultured fibroblasts with enucleated oocytes. The cells used in these studies were subjected to an extensive culture time, freezing and thawing, and clonal expansion from single cells prior to nuclear transfer. PCR and FACS analysis determined that the cloned offspring contained and expressed the H-transferase transgene. Microsatellite analysis confirmed that the clones were genetically identical to the boar. The cell culture and nuclear transfer procedures described here will be useful for applications requiring multiple genetic manipulations in the same animal. *Mol. Reprod. Dev. 60: 189–195, 2001.* © 2001 Wiley-Liss, Inc.

**Key Words:** nuclear transfer; porcine; culture; genetic manipulation

## INTRODUCTION

Cloning by nuclear transfer in domestic species was first accomplished by the fusion of sheep embryonic cells to enucleated unfertilized oocytes (Willadsen, 1986; Smith and Wilmut, 1989). These procedures were later successfully applied to cattle (Prather et al., 1987; Willadsen, 1989; Bondioli et al., 1990; Barnes et al., 1993; Stice et al., 1994), goats (Yong and Yuqiang, 1998), and pigs (Prather et al., 1989). Nuclear transfer with early embryonic cells did not have much application for the production of transgenic animals since the donor cells were not grown in culture and not readily available for genetic manipulation. The reports by Campbell et al. (1996) and Wilmut et al. (1997) that live sheep were born after nuclear transfer with donor nuclei from cultured cells including somatic cells of an adult animal, created a new opportunity for the production of transgenic animals including those with site specific genetic alterations (Schnieke et al., 1997; McCreath et al., 2000). The ability to produce pigs containing site specific genetic alterations induced by homologous recombination would have great value in the development of these animals for human therapeutic applications.



Live births of cloned pigs produced by somatic cell nuclear transfer have been reported by three independent groups (Betthauser et al., 2000; Polejaeva et al., 2000; Onishi et al., 2000). In the report by Onishi et al. (2000) early passage (three population doublings) fetal fibroblast nuclei were injected into the cytoplasm of enucleated oocytes. Polejaeva et al. (2000) produced cloned pigs utilizing granulosa cells from a primary culture (time in culture not specified) in a double nuclear transfer procedure. In a third report, fetal fibroblasts at passage 0 or 2 were fused to enucleated oocytes and resulted in live births (Betthauser et al., 2000). We report here the birth of live nuclear transfer piglets derived from fibroblasts isolated and cultured from a 4-month-old H-transferase transgenic male. The H-transferase transgenic male from which fibroblasts were isolated was an F1 progeny from a transgenic line previously reported (Costa et al., 1999). The donor cells used in these studies had been extensively manipulated in vitro prior to nuclear transfer.

## MATERIALS AND METHODS

### Manipulation and Culture Media

Oocyte recovery and all manipulations were conducted in Beltsville Embryo Culture Medium (BECM) (Pursel and Wall, 1996). Following fusion of enucleated oocytes and donor fibroblasts, nuclear transfer embryos were cultured in NCSU-23 medium (Petters and Wells, 1993).

### Superovulation of Gilts and Collection of Oocytes

Unfertilized oocytes were recovered from either prepubertal or cycling gilts. In the case of cycling gilts, estrus was synchronized by oral administration of 18 mg altrenogest (Regu-Mate, Hoechst) daily. Altrenogest was given 5–9 days depending on the stage of

Grant sponsor: The National Institutes of Standards and Technology

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Received 19 February 2001; Accepted 16 April 2001

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MOLECULAR REPRODUCTION AND DEVELOPMENT 62:300–306 (2002)

# Transgenic Pig Expressing the Enhanced Green Fluorescent Protein Produced by Nuclear Transfer Using Colchicine-Treated Fibroblasts as Donor Cells

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**ABSTRACT** Fetal-derived fibroblast cells were transduced with replication defective vectors containing the enhanced green fluorescent protein (EGFP). The transgenic cells were treated with colchicine, which theoretically would synchronize the cells into G2/M stage, and then used as donor nuclei for nuclear transfer. The donor cells were transferred into the perivitalline space of enucleated in vitro matured porcine oocytes, and fused and activated with electrical pulses. A total of 8.3% and 28.6% of reconstructed oocytes showed nuclear envelope breakdown and premature chromosome condensation 0.5 and 2 hr after activation, respectively. Percentage of pronuclear formation was 62.5, 12 hr after activation. Most (91.4%) of the 1-cell embryos with pronuclei did not extrude a polar body. Most (77.2%) embryos on day 5 were diploid. Within 2 hr after fusion, strong fluorescence was detectable in most reconstructed oocytes (92.3%). The fluorescence in all NT embryos became weak 15 hr after fusion and disappeared when culture to 48 hr. But from day 3, cleaved embryos at the 2- to 4-cell stage started to express EGFP again. On day 7, 85.8% of cleaved embryos expressed EGFP. A total of 9.4% of reconstructed embryos developed to blastocyst stage and 71.5% of the blastoctysts expressed EGFP. After 200 reconstructed 1-cell stage embryos were transferred into four surrogate gilts, three recipients were found to be pregnant. One of them maintained to term and delivered a healthy transgenic piglet expressing EGFP. Our data suggest that the combination of transduction of somatic cells by a replication defective vector with the nuclear transfer of colchicine-treated donors is an alternative to produce transgenic pigs. Furthermore, the tissues expressing EGFP from descendents of this pig may be very useful in future studies using pigs that require genetically marked cells. *Mol. Reprod. Dev. 62: 300–306, 2002.*


**Key Words:** nuclear transfer; enhanced green fluorescent protein; transgene; G2/M stage; pig

## INTRODUCTION

Successful somatic cell nuclear transfer provides a promising method to produce transgenic animals. This concept was strongly supported by generation of transgenic sheep (Schnieke et al., 1997), pigs (Park et al., 2001), and calves (Cibelli et al., 1998), and gene-targeted sheep (McCreath et al., 2000) and pigs (Lai et al., 2002), derived from nuclear transfer approaches by using transfected somatic cells. For pigs, somatic cell nuclear transfer has another special significance, as is it would allow the use of genetic modification procedures to produce tissues and organs from cloned pigs with reduced immunogeneticity for use in xenotransplantation. However, there are at least two obstacles

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David B. Carter is the recipient of a Pathobiology fellowship via funding from NIH.

Grant sponsor: F.B. Miller fund, Department of Animal Sciences, University of Missouri-Columbia (to H.T.C.); Grant sponsor: NIH DHHS (to R.S.P. and B.N.D.); Grant number: R01 RR13428; Grant sponsor: Food for the 21st Century; Grant sponsor: NIH DHHS (to D.B.C.); Grant number: T32 RR07004.

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Received 27 November 2001; Accepted 25 February 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mrd.10146

for using nuclear transfer approaches to produce transgenic animals. First, senescence of primary somatic cells in livestock species is generally seen following approximately 30 populations doubling ex vivo in non clonal cultures. Second, the efficiency of nuclear transfer in all species is very low. One of the many factors affecting the efficiency of nuclear transfer is the cell cycle phase of donor cells. Wilmut et al. (1997) using sheep mammary cells, stated that the donor cells for nuclear transfer must be in G0 of the cell cycle (quiescent phase). But Cibelli et al. (1998) showed that cycling cells, which contain cells in different cycle stages, could be successfully used for nuclear transfer in cattle. We argued that G2/M stage synchronized donor nuclei might represent an advantage, compared to other cell cycle-stages used in nuclear transfer (Prather, 2000), when considering nuclear-cytoplasmic synchronization of karyoplasts and cytoplasts. This argument was supported by some early studies on nuclear transfer using G2/M stage blastomeres as nuclear donors to produce cloned mice (Cheong et al., 1993, 1994) and sheep (Liu et al., 1997), and recent successes of using G2/M stage embryonic stem (ES) cells as donors to produce cloned mice (Wakayama et al., 1999; Amano et al., 2001; Zhou et al., 2001). We questioned whether differentiated somatic cells in G2/M stage could be used to produce cloned animals.

We, therefore, used a replication defective vector to transduce fetal fibroblasts, followed by treatment of colchicine, which theoretically would synchronize the cells into G2/M cell cycle stage, and subsequently used these cells as donor nuclei to conduct nuclear transfer, producing a transgenic cloned pig expressing the enhanced green fluorescent protein (EGFP).

## MATERIALS AND METHODS

### Preparation of Donor Cells

A day 35 crossbred porcine fetus was obtained from a pregnant gilt. The tissue was cut into small pieces with fine scissors. The cells were incubated for 30 min at 37°C in PBS containing 0.05% trypsin and 0.02 mM EDTA, then the suspension was centrifuged. The cell pellet was resuspended and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 0.1 mM Na-pyruvate, 75 μg/ml penicillin G, 50 μg/ml streptomycin, and 15% (v:v) FCS. The cells were passaged seven times. To transduce the fetal-derived fibroblasts, a replication-defective vector based on Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus (VSV-G) was used. The retroviral vector, which was kindly provided by Dr. A.W.S. Chan, consisted of a long terminal repeat, neomycin resistant gene and an EGFP gene under the control of the CMV promoter (Chan et al., 2001).

Fibroblasts were transduced by co-incubation with the vector overnight. G-418 selection was started the following day and was performed for 13 days. To obtain the G2/M stage donor cells, medium was removed and replaced with medium containing colchicine (1.0 μM) and cultured for 24 hr (Boquest et al., 1999). The synchronized cells were harvested by standard-trypsinization, and subsequently, 50 μl aliquots containing 1,500–3,000 cells were frozen in culture media supplemented with 10% DMSO. Before microinjection, donor cells were thawed at 37°C, and 200 μl FCS was added and cultured for 30 min. Then 800 μl DMEM supplemented with 15% FCS was added, and the sample was centrifuged at 500*g* for 5 min. The supernatant was removed and 50 μl TCM-199 supplemented with HEPES was added to resuspend the cells.

### Flow Cytometric Analysis of Cell Cycle Stage

Colchicine-treated fetal fibroblasts were analyzed for DNA content by ethanol fixation and staining with propidium iodide (PI) using a method described by Crissman and Steinkamp (1982). The largest cells were analyzed using Becton and Dickinson flow cytometer as described by Crissman and Steinkamp (1982). Percentages of cells existing within the different phases of the cell cycle were calculated using 'Cell Quest' program gating on G0/G1 and G2/M cell populations visualized using the scatter plot of red fluorescence.

### Preparation of Recipient Oocytes

Prepubertal porcine ovaries were obtained from an abattoir and transported to the laboratory in a thermos filled with saline maintained at 30–35°C. Follicular fluid from 3–6 mm antral follicles was aspirated by using an 18-gauge needle attached to a 10 ml disposable syringe. Cumulus–oocytes complexes (COCs) with uniform cytoplasm and several layers of cumulus cells were selected and rinsed three times in TL-HEPES plus PVA. Approximately 50–70 COCs were transferred into each well of four-well multidish containing TCM-199 medium supplemented with PVA (0.1%), D-glucose (3.05 mM), sodium pyruvate (0.91 mM), penicillin (75 μg/ml), streptomycin (50 μg/ml), cysteine (0.57 mM), LH (0.5 μg/ml), FSH (0.5 μg/ml), and EGF (10 μg/ml), covered with mineral oil. The oocytes were matured for 42–44 hr at 39°C, 5% $CO_2$ in air.

### Nuclear Transfer

After 44 hr of oocyte maturation, oocytes were freed from cumulus cells by vigorous vortexing for 4 min in TL-Hepes supplemented with 0.1% PVA and 0.1% hyaluronidase. Cumulus-free (denuded) oocytes were enucleated by aspirating the first polar body (PB) and adjacent cytoplasm in enucleation medium with a glass pipette 30 μm in diameter. The largest cells (22–28 μm in diameter) (Fig. 1A) from the colchicine-treated population were chosen as the G2/M stage donor cells to be injected directly into the perivitelline space of the oocyte. Injected oocytes were placed between 0.2 mm diameter platinum electrodes 1 mm apart in fusion/activation medium. Fusion/activation was induced with 2 DC pulses (1 sec interval) of 1.2 kV/cm for 30 μsec on a BTX Elector-Cell Manipulator 200 (BTX, San

Diego, CA). Parthenogenetic embryos were derived by exposing oocytes to the same electrical pulses as were the nuclear transfer embryos. The medium used for enucleation was tissue culture medium (TCM) 199 supplemented with HEPES, 0.3% BSA, and 7.5 μg/ml cytochalasin B (CB), and the medium for injection was the same medium without CB. The medium used for fusion and activation consisted of 0.3 M mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.5 mM HEPES.

### Evaluation of Nuclear Remodeling Events of Donor Nucleus

To observe nuclear envelope broke down (NEBD), premature chromosome condensation (PCC) PB and pronuclear formation 36, 42, and 56 embryos were fixed at 0.5, 2, and 12 hr after fusion and activation, respectively. Fixation was conducted by mounting oocytes on glass slides under coverslips and fixed in ethanol: acetic acid (3:1) for 24 hr. Oocytes were then stained with 1% aceto–orcein and evaluated by using Hoffman modulation contrast optics.

### Evaluation of In Vitro Development and EGFP Expression of Nuclear Transfer Embryos

A total of 297 embryos were cultured in NCSU with 0.4% BSA for 7 days. The embryos were checked with an ultraviolet microscope on 2, 15, 48, 72 hr and on day 7 to determine if EGFP was being expressed. Cleavage and blastocyst rate was checked at 48 hr and on day 7, respectively. Blastocysts were stained with Hoechst 33342 to count the number of nuclei. In another two replicates, 22 morula or early stage blastocysts cultured to day 5 were treated with 0.08 μg colcemid for ploidy analysis with the procedure described by Burgoyne (1993).

### Embryo Transfer

The 1-cell stage embryos were surgically transferred into oviduct of the non bred day 0 or 1 recipient pigs to allow the pigs to continue pregnancy. Two surrogates were transferred with nuclear transferred embryos and parthenogenetic embryos. Two surrogates received only nuclear transfer embryos, but treated with 3 mg of estradiol cypionate (ECP, Pharmacia & Upjohn Co., Kalamazoo, MI) on day 12 after embryo transfer.

### Parturition

Parturition was induced on day 112 of gestation using 10 mg of dinoprost (Lutalyse, Pharmacia & Upjohn, Kalamazoo, MI) followed in 6 hr by 10 mg of estradiol benzoate. At the same time as the Lutalyse, 5 mg of dexamethasone was administered to the sow to accelerate fetal lung maturation and improve respiratory function after birth. Thirty hours after induction, on day 114, the sow showed few signs of impending parturition and had minimal mammary gland development. A litter of one live piglet was subsequently delivered by cesarean section. Immediately after removal from the uterus, the piglet was placed on a heated table, rubbed vigorously, and oxygen was administered. One hour after delivery, the piglet was weighed and received a physical examination. The piglet was then administered 15 ml of pooled colostrum to ensure adequate immunoglobulin intake before the piglet was placed with the sow.

## RESULTS

After being transduced by the retroviral vector and followed by G-418 selection for 13 days, all survival cells were confirmed to be expressing EGFP under ultraviolet microscopy. The green cells were subcultured and fast proliferation was observed. After treating the cells with colchicine for 24 hr, about 23.9% (125/521) cells were with size of above 22 μm in diameter (Fig. 1A,B), which were classified into the largest population to be used as donors for nuclear transfer in this experiment. To identify possible cell cycle stages of these donors, the largest cells were subjected to flow cytometric analysis. 70.5% (840/1192) of these cells were in G2/M stage, 9.7% (116/1192) in S stage, and 18.8%(224/1192) in G0/G1 stage (Fig. 2).

In three replicates, the nuclear remodeling events were evaluated within 12 hr after activation and fusion. A total of 8.3% (3/36) and 28.6% (12/42) of reconstructed oocytes showed NEBD and PCC 0.5 and 2 hr after activation, respectively. Percentage of pronuclear formation was 62.5% (35/56) 12 hr after activation. Out of the 1-cell embryos with pronuclei, 8.6% (3/35) extruded a PB, most of them (31/35, 88.5%) formed one pronucleus. In another two replicates, we analyzed the karyotype of the embryos in morula or early blastocyst stage on day 5. Out of 22 embryos, 17 were diploid (77.2%), 2 were tetraploid, and 3 were mosaic embryos with both diploid and tetraploid cells.

In another five replicates, we monitored the in vitro development and EGFP expression of the reconstructed embryos. The fusion rate, cleavage rate, blastocyst formation rate, and the average nuclear number of blastocysts were 81.6% (297/364), 59.6% (177/297), 9.4% (28/297), and 25.7% (719/28), respectively.

Within 2 hr after fusion, strong fluorescence was detectable in 36 of 39 reconstructed oocytes (92.3%) (Fig. 3A). The fluorescence in all NT embryos became weak 15 hr after fusion and disappeared when culture to 48 hr. But from day 3, cleaved embryos at the 2- to 4-cell stage started to express EGFP again (Fig. 3B). On day 7, out of 177 cleaved embryos, 152 expressed EGFP (85.8%). The EGFP expressing embryos were in the range of 2-cell to blastocyst stage (Fig. 3C,D). We obtained 28 blastocysts out of 297 cultured NT embryos, and of them, 20 expressed EGFP (71.5%).

As shown in Table 1, two surrogates received nuclear transfer embryos and parthenogenetic embryos. One of the two recipients receiving 22 NT embryos and 15 parthenogenetic embryos established pregnancy. Tracing the fetal development by transabdominal ultrasound at 7-day intervals, we found that uterine enlargement occurred as normal and pregnancy vesicles could be observed as early as 24 day. However, these

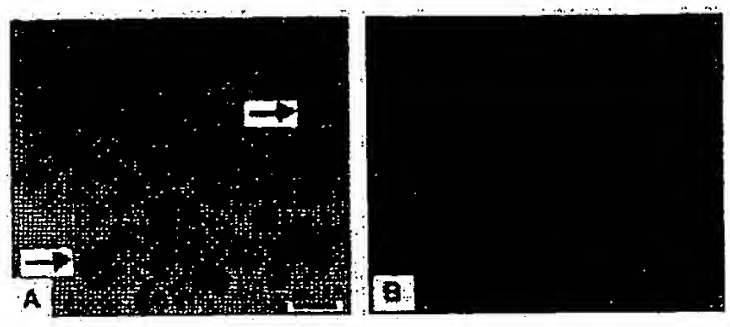


Fig. 1. A: Fibroblast population treated with colchicine. The largest cells (arrows) were used as G2/M stage donors. B: Donors expressing EGFP under ultraviolet light.

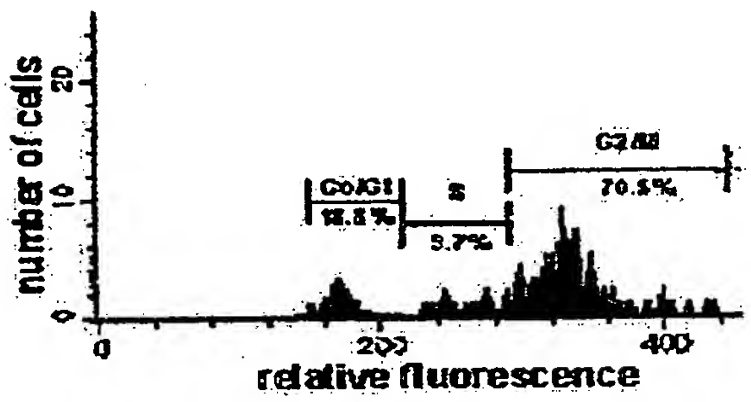


Fig. 2. Flow cytometric analysis of cell cycle stages in the largest cell population after treated with colchicine. A total of 70.5% of the biggest cells were in G2/M stage, 18.8% in G0/G1 stage, and 9.7% in S stage.

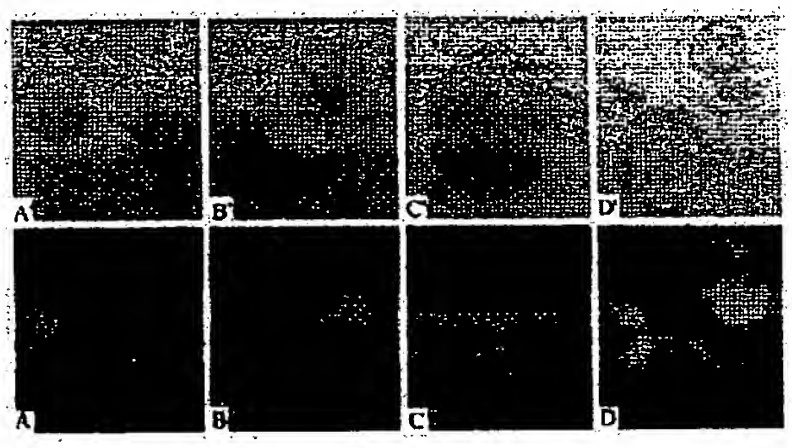


Fig. 3. A: Two hours after activation and fusion, reconstructed oocytes expressing EGFP. B: Three days after culture, EGFP expression was found in 2- and 4-cell stage embryos. C: Seven days after culture, EGFP expression occurred in morula stage embryos. D: Seven days after culture, EGFP expression was found in 2-cell and blastocyst stage embryos.

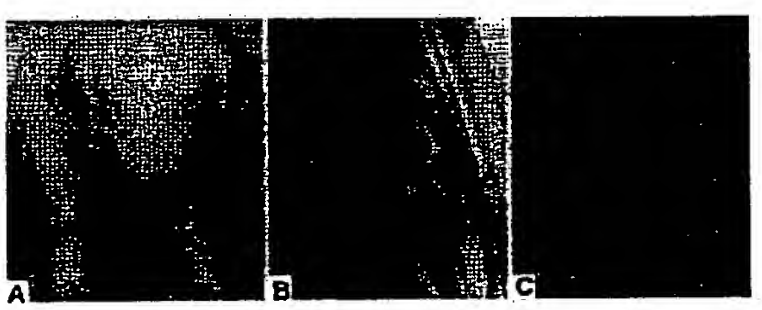


Fig. 4. A: Cloned piglet expressing EGFP. Yellow coloration of the snout, hoof wall of each toe was obvious. B: Skin and hair of the cloned pig under bright field. C: Skin and hair of the cloned pig emitted fluorescence under ultraviolet light.

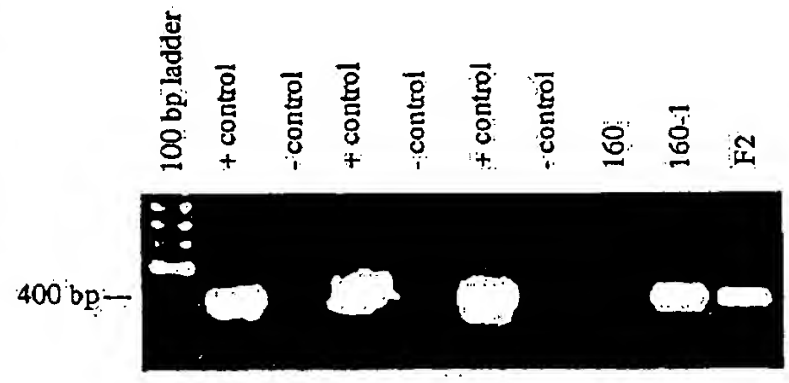


Fig. 5. EGFP genotyping by PCR. Lane1, ladder; lane 2, positive control; lane 3, negative control; lane 4, surrogate; lane 5, cloned piglet; lane 6, donor cells. Forward primer: 5′-CGC ACC ATC TTC TTC AAG GAC GAC-3′; Reverse primer: 5′-AAC TCC AGC AGG ACC ATG TGA TCG-3′. Reaction conditions: 94°C, 5 min; 94°C, 45 sec; 61°C, 30 sec; 72°C, 45 sec (32 cycles); 72°C, 5 min; 4°C hold.

began to degenerate and were no longer visible by day 40. Subsequently, the surrogate gilt returned to estrus on day 69. Another two surrogates received only nuclear transfer embryos and treated with estrogen 12 days after embryos transfer. One that received 58 embryos was diagnosed as pregnant on day 24, but returned to estrus on day 31. The other surrogate receiving 70 NT embryos maintained pregnancy to term and delivered a female piglet (Fig. 4A) on day 114 (day 0 = date of estrus of surrogate) by cesarean section. The birthweight of the piglet was 1,256 g. This is similar to average birth weights of 1,450 g in our sow herd. Microsatellite analysis using three polymorphic markers confirmed that the piglet was derived from the transfected cell line, FF2-EGFP (Table 2). The piglet clearly expressed the EGFP. Tissues from the skin, including hair emitted green fluorescence when observed under an ultraviolet light (Fig. 4B,C). The epithelial tissues of the snout, oral, and nasal mucosa, and coronary band and hoof wall of each limb displayed a brilliant yellow coloration (Fig. 3A). This may be caused by the accumulation of a high density of keratinized epithelial cells expressing EGFP. PCR results confirmed that the genome of the piglet contained the EGFP gene (Fig. 5). The karyotype of the cells isolated from the piglet's ear tissue was diploid (data not shown). Physical examination of the piglet shortly after birth revealed no abnormalities. A venous blood sample was obtained from the piglet at 24 hr of age and a routine complete blood count, and serum biochemical panel were within normal limits.

## DISCUSSION

To have more chance to use G2/M stage cells as donors in this experiment, two measures as reported by Wakayama et al. (1999) were taken. First, we treated the cells with a microtubule-disrupting agent, colchicine, which arrested the population of cells at G2/M before nuclear transfer. Previously, we showed that after treatment with colchicine, porcine fibroblasts could be efficiently synchronized into the G2/M cell-cycle stage (Boquest et al., 1999). Secondly, we chose

**TABLE 1. Nuclear Transfer Embryos Transferred to Surrogate Gilts**

| Pig # (estrus) | Embryos transferred | Results | Helper |
|---|---|---|---|
| Y78 (d1) | 58 | Pregnant<br>Lost on day 25–31 | Estradiol |
| O160 (d0) | 70 | One piglet delivered | Estradiol |
| Y73 (d1) | 22 | Pregnant<br>Cycled on day 69 | 15 Parth. |
| Y95 (d1) | 50 | Not pregnant<br>Cycled on day 23 | 15 Parth. |

Estradiol, surrogates were injected with estradiol on day 12 after embryo transfer. Parth., parthenogenetic embryos were co-transferred with nuclear transfer embryos.

the largest cells from the colchicine-treated population, which are double in size compared to the smallest cells. According to previous studies, the cells with such a relative large cytoplasmic volume are typical of those that have advanced beyond the G1-phase, and whose cytoplasmic swelling is in preparation for division (Steen and Lindmo, 1978; Zetterberg and Larsson, 1995). To further characterize these cells, we checked the cell cycle stage of these biggest cells with flow cytometric analysis. As shown in Figure 2, more than 80% of the biggest fibroblasts was beyond the G1 cell cycle stage. Therefore, results obtained in present experiment could mainly represent the characterization of nuclear transfer embryos derived from G2/M cell cycle donors.

Enucleation of in vitro matured metaphase II oocytes was performed without staining the chromatin, which can be detrimental to development (Dominko et al., 2000; Tao et al., 2000). In previous experiments, the success of enucleation varied between 85 and 90%. Due to this high enucleation efficiency, we decided to use "blind enucleation" for our NT experiments. The low rate of NT embryo development in the pig has been suggested to result from inadequate artificial activation of the oocytes. The strategy of Polejaeva et al. (2000), using double nuclear transfer, the second round being the transfer of NT pronuclei into enucleated IVF zygotes, was meant to circumvent the need for an artificial activation protocol. Betthauser et al. (2000) exposed the porcine cytoplasmic hybrids to ionomycin according to a bovine activation protocol. In our experiments, the reconstructed oocytes were electrically fused and simultaneously activated in activation medium with a high calcium concentration (1 mM). We obtained a high fusion, pronuclear formation, and cleavage percentage.

**TABLE 2. Microsatellite Analysis of Donor Cell Lines, Surrogate Gilt, and Offspring**

| | Marker | | |
|---|---|---|---|
| Tissue | S0097 sizes (bp) | SW902 sizes (bp) | S0301 sizes (bp) |
| FF2-GFP | 207 | 201/207 | 257/262 |
| O160 (surrogate) | 207 | 199/203 | 255/262 |
| 0160-1 (offspring) | 207 | 201/207 | 257/262 |

To clarify the concern, if embryos with normal ploidy could be obtained by using cochicine-treated donors, the morphological events that occur to the donor nucleus were studied. In a previous experiment, G2/M stage donor cells were injected directly into non activated matured oocytes, in which maturation-promoting factor (MPF) levels were high, the donor NEBD and PCC was found in most reconstructed oocytes. As a result of NEBD having allowed access of licensing factors to the DNA, nuclei would undergo DNA replication before the first cleavage. We found that 80.6% of the reconstructed oocytes, which formed pronucleus-like structures, extruded polar bodies (Lai et al., 2001). In the present experiment, since we conducted the fusion and activation simultaneously, MPF levels might be reduced at the time when the donor nuclei merged into the cytoplasm of oocytes. Therefore, we hypothesized that most donor nuclei, in both G0/G1 and G2/M stage, would not undergo PCC and DNA replication. The embryos derived from G2/M stage would remain diploid without emission of a PB before the first cleavage. In this experiment, by using colchicine-treated donors, only 8.3% and 28.6% of reconstructed oocytes showed PCC 0.5 and 2 hr after activation, respectively. Out of the 1-cell embryos with pronuclei 12 hr after activation, only 8.6% extruded a PB, most of them (88.5%) formed one pronucleus. However, most embryos were diploid (77.2%). Those results supported our hypothesis.

We monitored the in vitro development and EGFP expression of the reconstructed embryos. Within 2 hr after fusion, strong fluorescence was detectable in reconstructed oocytes. The fluorescence in these reconstructed oocytes was probably not derived from the transcription of the donor cell genome; instead, it might be from cytoplasm of the donor cell, which contained EGFP mRNA as well as protein. Since the fusion method was employed in this experiment, any cytoplasmic material from the donor cells would be fused into the cytoplasm of oocytes. From 15–48 hr after fusion, the fluorescence in NT embryos became weaker or disappeared. But from day 3, cleaved embryos at the 2- to 4-cell stage started to express EGFP again. The above observation suggests that the G2/M stage donor genome stopped transcription after activation and fusion because the nuclei of the donor cells had been reprogrammed by the cytoplasts. After culture to day 3, transcription was initiated again. In swine, the transition to zygotic control of development occurs during the 4-cell stage, which is about 72 hr after fertilization. But we found some 2-cell stage embryos also expressed EGFP after 3 days of culture. This suggests that the initiation of transcription of maternal control is not only embryo-stage dependent, but may be embryo-age dependent.

The formation of EGFP-expressing embryos with normal ploidy suggests that G2/M stage donors are able to lead to normal embryo development to term. The percentage of blastocyst formation and the average nuclear number of the blastocysts were not high. To

overcome this problem, we limited the culture time to less than 24 hr before embryo transfer. In the report of Onishi et al. (2000), the percentage of blastocysts was only 2.4%, but a recipient pig established pregnancy and a cloned piglet developed to term.

Pregnancy initiation in the pig requires a critical minimum signal from the embryos to the mother around day 12 of gestation, and this has been estimated to be four embryos (Polge et al., 1966). To address this problem, two measures were taken to raise the pregnancy efficiency. First, we transferred the NT embryos with parthenogenetically-activated embryos, which were thought to enhance the signal for maternal recognition of pregnancy and would degenerate later in gestation because of genomic imprinting. Although pregnancy was detected in one of the two recipients, unfortunately, all fetuses gradually degenerated 40 days after embryo transfer. In this pig, only parthenogenetic embryos might have initiated the pregnancy. Second, estradiol, which is supposed to be a signal to stimulate the development of uterus for accepting the implantation of fetuses, was injected on day 12 after embryo transfer. By this method, both of the recipients were found to be pregnant, and one of them maintained to term. A healthy piglet with normal phenotype including diploid karyotype and expression of EGFP was successfully obtained. This cloned piglet with normal phenotype is unique compared to the report of Ono et al. (2001) in which two dead cloned mice with abnormal phenotypes were obtained when M stage fibroblasts were used as donor cells, and only when serial nuclear transfer was carried out were, they able to obtain normal cloned mice.

Our results suggest that colchicine-treated somatic cell nuclei, most of which in G2/M cell cycle stage can be reprogrammed in enucleated in vitro matured oocytes and direct the reconstructed embryo to develop blastocyst stage, and probably to viable cloned piglets with normal ploidy. The success of this experiment is significant in two ways. First, the procedures established in this experiment would be an alternative approach to produce cloned pigs supplying tissues and organs for xenotransplantation. Second, descendents of the transgenic pig expressing EGFP will likely provide a variety of genetically marked tissues, which would be very useful for basic research where such marked cells are required.

## ACKNOWLEDGMENTS

The authors thank A.W.S. Chan for the generous gift of replication defective retrovirus containing the EGFP gene, Edmund Rucker and Dave Durtshi for running the microsatellites and the PCR for EGFP on the resulting piglet, and U.S. Pig Genome Coordinator, Max Rothschild for the primers for the microsatellites. The authors also thank Ed Brown and Daniel Liske for care of the surrogate gilts during gestation, Tom Cantley for help with surgical embryo transfers, and Kristin Whitworth, Rami Woods, Zhixin Wei, Jennifer Luth, Lisa Overman, Ryan Cabot, and Dave Wax for helping to care for the piglets after delivery. We acknowledge funding from the F.B. Miller fund (Department of Animal Sciences, University of Missouri-Columbia) to H.T.C., funding from the NIH DHHS via R01 RR13428 to R.S.P. and B.N.D., and Food for the 21st Century.

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# Production of gene-targeted sheep by nuclear transfer from cultured somatic cells

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It is over a decade since the first demonstration that mouse embryonic stem cells could be used to transfer a predetermined genetic modification to a whole animal[1]. The extension of this technique to other mammalian species, particularly livestock, might bring numerous biomedical benefits, for example, ablation of xenoreactive transplantation antigens, inactivation of genes responsible for neuropathogenic disease and precise placement of transgenes designed to produce proteins for human therapy. Gene targeting has not yet been achieved in mammals other than mice, however, because functional embryonic stem cells have not been derived. Nuclear transfer from cultured somatic cells provides an alternative means of cell-mediated transgenesis[2,3]. Here we describe efficient and reproducible gene targeting in fetal fibroblasts to place a therapeutic transgene at the ovine α1(I) procollagen (*COL1A1*) locus and the production of live sheep by nuclear transfer.

We previously showed that transfection of fetal fibroblasts with nuclear transfer offers an efficient and practical method of producing sheep carrying randomly integrated transgenes[4], and similar work has been reported in cattle[5]. Gene targeting is a more powerful method of genetic manipulation and requires essentially the same procedures of transfection and drug selection of cultured cells. Although experimental gene targeting was first carried out in somatic cell lines[6–8], the use of embryonic stem (ES) cells now predominates; however, there has been no definitive comparison of gene targeting efficiency in primary somatic cells and ES cells (but see ref. 7 for review). We wished to determine whether practically useful gene targeting could be achieved in primary ovine fetal fibroblasts, and whether these cells could produce viable animals by nuclear transfer. The ovine *COL1A1* gene represented a suitable target with which to establish gene targeting in fetal fibroblasts for three reasons. First, we expected that gene-targeting events would be very rare compared with random integrations. *COL1A1* is highly expressed in fibroblasts, allowing promoter-trap enrichment of gene-targeting events. Second, few ovine genes have been cloned and characterized. *COL1A1* is well studied and highly conserved in several species, facilitating molecular cloning and construction of ovine gene-targeting vectors. Third, mutations in *COL1A1* can cause connective tissue disorders in humans, for example, *osteogenesis imperfecta*[9,9]. The ability to generate models of human genetic disorders by gene targeting in animals other than mice might be valuable for clinical research; however, we chose to target a site that would not significantly affect type I collagen protein function or expression to avoid affecting fetal development.

We used two gene-targeting vectors to target ovine *COL1A1* (Fig. 1). Each vector incorporated two regions of *COL1A1* homology, derived from a contiguous fragment of Poll Dorset fetal fibroblast[3] PDFF2] genomic DNA. COLT-1 was designed to insert a promoterless *neo* selectable marker between the *COL1A1* translational stop and polyadenylation signal, such that transcription of the targeted locus resulted in a bicistronic messenger RNA. An internal ribosomal entry site (IRES)[10] immediately 5′ of *neo* facilitated translation. COLT-2 had the same structure, but included a transgene as a separate transcription unit located 3′ of *neo*. This transgene, termed AATC2, comprised human α1-antitrypsin (AAT) complementary DNA within an ovine β-lactoglobulin (BLG) expression vector designed to direct expression in the lactating mammary gland[11].

We transfected COLT-1 DNA into early passage PDFF2 female and PDFF5 male ovine primary fetal fibroblasts; we transfected COLT-2 DNA into PDFF2 cells. Stable G418 resistant clones

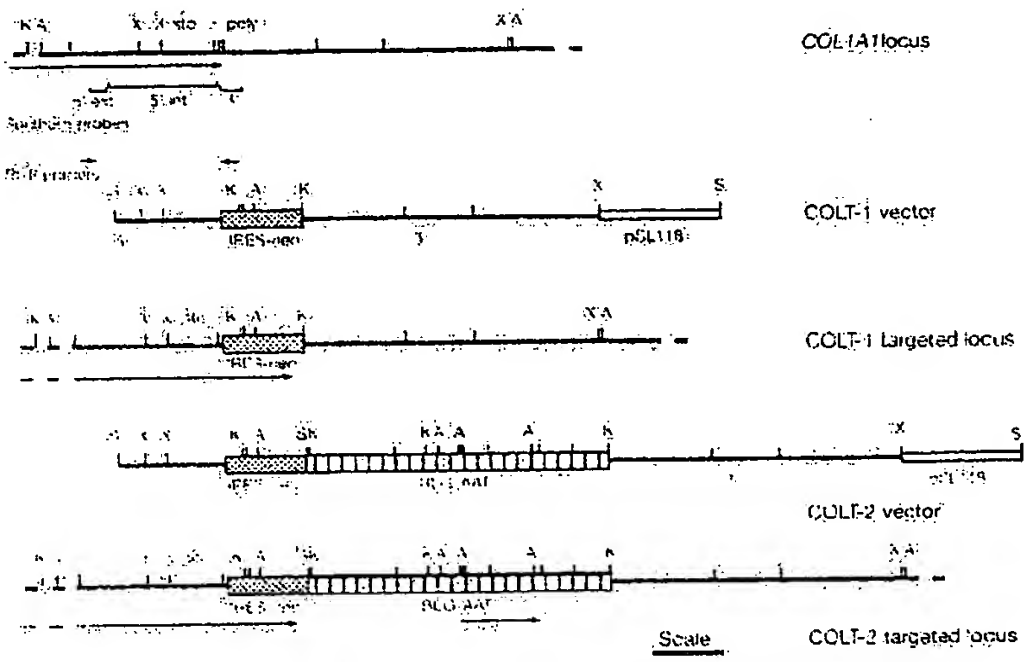


**Figure 1** [illegible] cassette is a shaded box, the BLG driven AAT transgene by a striped box, and the bacterial vector pSL1180 by an open box. The direction and predicted extent of the IRES-neo bicistronic mRNA and the AAT transgene mRNA are shown as [illegible]. The scale bar represents 2 kb. Restriction enzyme sites: A, AspI; B, BamHI; [illegible] Sall; X, XhoI.

were derived. About 30 days of culture elapsed between fetal disaggregation and cryopreservation of gene-targeted cell clones. DNA samples of each cell clone were initially screened by polymerase chain reaction (PCR) using primers designed to amplify a 3.4-kilobase (kb) fragment across the 5′ junction of the targeted locus (Fig. 1). In each case, a high proportion of G418 resistant cell clones were found to have undergone gene targeting (5 out of 36 PDFF2 COLT-1, 4 out of 56 PDFF5 COLT-1, and 46 out of 70 PDFF2 COLT-2 cell clones analysed). We called COLT-1 cell clones 'PDCOL' and COLT-2 cell clones 'PDCAAT'. The DNA sequence of PCR products amplified from three PDCOL and two PDCAAT cell clones was determined across the 5′ junction; each was consistent with gene targeting (data not shown).

Figure 2a shows Southern analysis of the 5′ junctions of a series of PDCAAT cell clones. All samples showed the presence of a 7-kb *Bam*HI fragment from the normal *COL1A1* locus. Each clone identified as positive by PCR also showed a diagnostic 4.7-kb *Bam*HI fragment spanning the 5′ junction of the COLT-2 targeted locus. This is consistent with the presence of one targeted and one normal *COL1A1* allele. PDCAAT cell clones 87 and 99 also showed the presence of additional bands, indicating additional integrations of COLT-2.

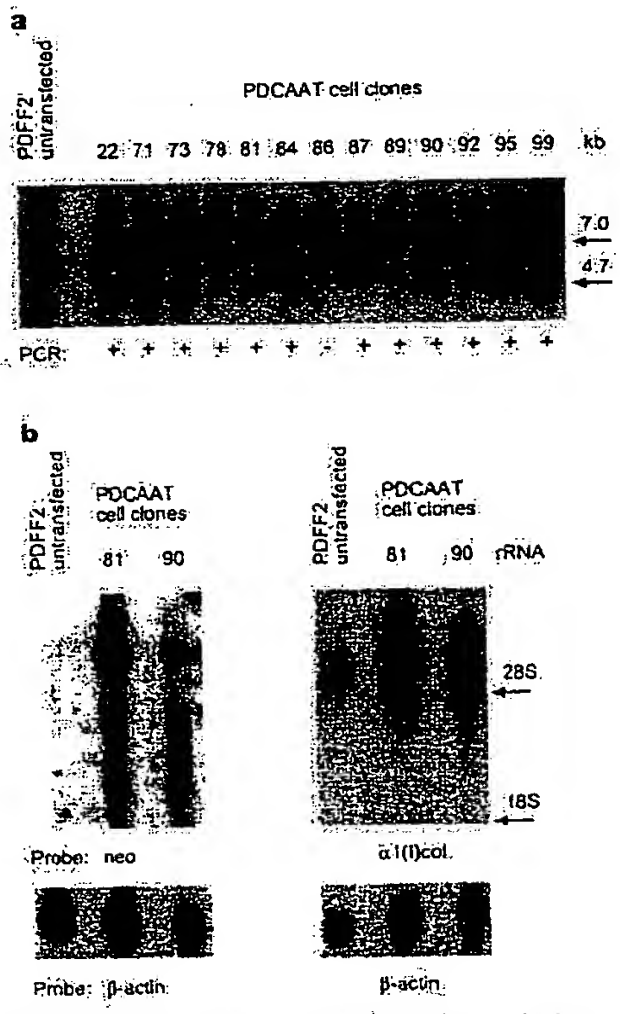


**Figure 2** Analysis of COLT-2 transfected (PDCAAT) cell clones. **a**, Southern analysis. Each lane contains *Bam*HI-digested genomic DNA from cell samples hybridized with a 3 kb *COL1A1 Sal*I–*Sst*I fragment corresponding to the 5′ homologous arm of COLT-1 and COLT-2 (see Fig. 1). The cell clone is indicated above each lane and results of the PCR screen are shown below each lane. The positions of the 7-kb *Bam*HI fragment from the non-targeted ovine *COL1A1* locus and the 4.7 kb *Bam*HI fragment from the COLT-2 targeted locus are marked. **b**, Northern analysis. Each lane contains 10 μg total RNA extracted from PDFF2 cells and targeted cell clones PDCAAT81 and PDCAAT90 as indicated. Duplicate blots were hybridized with a *neo* probe (upper left), and full-length human α1(I) procollagen cDNA (upper right). The positions of the 28S and 18S rRNA bands are indicated. The lower two autoradiographs show rehybridization of the same blots with a β-actin cDNA as an indication of the amount of total RNA loaded.

PDFF5 cells were also targeted at high frequency with COLT-1. PDFF2 and PDFF5 cells have different parentage from within the PPL outbred flock of Poll Dorset sheep. This indicates that it may not always be essential to isolate DNA from the same individual, or an animal of the same inbred strain, to achieve efficient gene targeting[12]; however, the degree of sequence divergence, if any, between the targeted *COL1A1* alleles in these cells has not yet been determined.

Northern analysis of cell clones PDCAAT 81 and 90 is shown in Fig. 2b. Hybridization with human α1(I) procollagen cDNA detected a 4.8-kb mRNA species in both non-transfected cells and PDCAAT cell clones, consistent with expression of normal *COL1A1*. A larger species of about 6.8 kb was present only in the PDCAAT cell clones, consistent with a bicistronic *COL1A1*–IRES–*neo* fusion mRNA. Hybridization of the same RNA samples with a *neo* probe also detected a 6.8-kb mRNA in the targeted clones, again consistent with a bicistronic mRNA. These results confirmed that gene targeting had occurred. This analysis also showed that, unlike mouse, rat and human, which express two endogenous α1(I) procollagen mRNA species from different polyadenylation sites[13,14], sheep express a single mRNA species.

Although these experiments were designed to avoid disruption of *COL1A1* gene expression, the mRNA from the targeted locus is less abundant than the wild type (Fig. 2b). Whether this reflects different mRNA stability or transcriptional activity has yet to be determined. However, elements which affect transcription have been identified at the 3′ end of *COL1A1* in other species[15,16], and targeted DNA insertion may affect their function.

We carried out northern analysis to determine whether placement of the AATC2 transgene adjacent to the highly expressed *COL1A1* gene resulted in aberrant expression of the BLG promoter in fibroblasts. Hybridization with human AAT cDNA failed to detect AAT mRNA expression in either PDCAAT81 or 90 cells (data not shown), indicating no apparent loss of BLG promoter specificity.

Four targeted cell clones, all derived from PDFF2 female cells (PDCOL6, PDCOL13, PDCAAT81 and PDCAAT90), were selected for nuclear transfer on the basis of their vigour and normal metaphase chromosome number. Nuclear transfer was carried out on twelve occasions and the results are summarized in Table 1. Fourteen lambs were live-born: seven died within 30 hours of birth, and one each after 3 days, 8 days, 7.5 weeks and 12 weeks. Three lambs are currently alive and thriving at almost one year of age. The first two live-born lambs are shown in Fig. 3.

*Post mortem* examination of lambs that died *in utero* or after birth revealed a range of abnormalities. Although there was no consistent pattern, we observed a high incidence of kidney defects (frequently renal pelvis dilation), liver and brain pathology. These findings are similar to a previous nuclear transfer study using the same cells[7].

**Table 1 Summary of nuclear transfer results**

| Nuclear donor cells | PDCOL 6 | PDCOL 13 | PDCAAT 81 | PDCAAT 90 |
|---|---|---|---|---|
| Reconstructed embryos | 109 | 154 | 71 | 82 |
| Embryos recovered from [illegible] | 104 | 149 | 62 | 78 |
| Embryos developed to morula or blastocyst | 14 | 43 | 4 | 11 |
| Embryos transferred to final recipients | 11 | 43 | 4 | 13 |
| Final recipients | 8 | 32 | 3 | 10 |
| Fetuses at day 60 | 5 | 10 | 2 | 2 |
| Live born lambs | 4 | 8 | 2 | 2 |
| Lambs alive beyond 1 week | 2 | 3 | 0 | 1 |
| Lambs alive beyond 6 months (birth ID) | 1 (ID 990502) | 1 (ID 990504) | 0 | 1 (ID 990507) |

and are therefore probably due to some aspect of the cell treatment or nuclear transfer procedure and not a consequence of gene targeting *per se*. Several researchers have reported developmental abnormalities associated with somatic cell nuclear transfer[1,17,18]. Although there is some indication that inappropriate expression of imprinted genes may be involved[19], definitive investigations have yet to be carried out. Understanding and rectifying this problem is a continuing priority.

Figure 4 shows Southern analysis of 5′ and 3′ junctions of the *COL1A1* targeted locus in representative nuclear transfer lambs. Hybridization with the same 5′ and 3′ probes used to analyse PDCAAT cell clones revealed fragments consistent with the presence of one targeted and one normal *COL1A1* allele in the two lambs shown. This was confirmed using a 5′ probe external to the vector.

Sixteen lambs and fetuses were analysed by Southern blot, of which fifteen confirmed the presence of a targeted allele. One lamb (990504), derived from PDCOL13, showed only the normal *COL1A1* gene, which probably indicates that the cell isolate was oligoclonal. Non-transfected cells have been detected within some G418 selected cell isolates in previous experiments (unpublished data).

Lamb 990507 derived from clone PDCAAT90 was hormonally induced to lactate and milk samples analysed by western blotting (data not shown). AAT was detected at a concentration of 650 μg ml$^{-1}$, which compares favourably with the highest level previously reported for an AAT cDNA transgene in sheep carrying multiple random gene inserts (18 μg ml$^{-1}$)[20]. This indicates that the *COL1A1* locus supports transgene expression even though it is not actively expressed in mammary epithelium[21].

We have shown that gene targeting can be carried out efficiently in somatic cells and that viable animals can be produced by nuclear transfer. We have also obtained preliminary data (that is, PCR fragment size and sequence) indicating similarly efficient targeting at the α-1,3-galactosyl-transferase locus in porcine fibroblasts (unpublished data). Notably, the use of nuclear transfer does not require embryonic stem or embryonic germ cells, and circumvents the generation of chimaeric animals, which would be costly and time consuming in livestock. Fibroblasts are also being used in clinical trials to provide a protein production system after *ex vivo* gene therapy in human patients[22], and it has been suggested that the introduction of therapeutic transgenes by homologous recombination could avoid undesirable effects arising from random integration[23]. Nuclear transfer in animals such as sheep provides a rigorous means of testing the suitability of specific loci for transgene placement. If the *COL1A1*-targeted sheep continue to show no locus-related deleterious effects, this would indicate that this target locus may provide a permissive and benign environment for the insertion of therapeutically useful genes. □

**Figure 3** Gene-targeted lambs. Lambs 990502 and 990503, both derived by transfer of nuclei from gene-targeted cell clone PDCOL6.

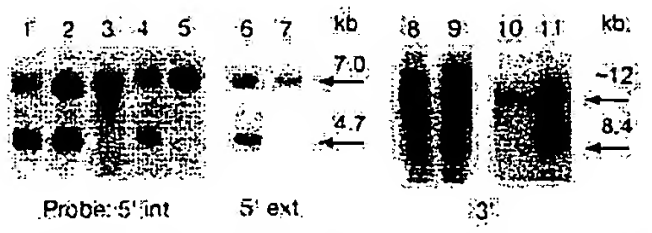


**Figure 4** Southern analysis of nuclear transfer lambs. Lanes 1—7 show *Bam*HI-digested genomic DNA samples hybridized with 5′ internal and external probes as indicated. [illegible] kb *Bam*HI fragment from the non-targeted ovine *COL1A1* locus, and [illegible] kb fragment from the COLT-1 and COLT-2 targeted locus. Lanes 8–11 show *Kpn*I [illegible] digested genomic DNA samples hybridized with the 3′ probe. Arrows indicate the ~12-kb *Kpn*I fragment from the non-targeted locus and the 8.4-kb *Kpn*I. [illegible] from the COLT-1 and COLT-2 targeted locus. Lanes 1, nuclear transfer lamb [illegible] from clone PDCOL6; lane 2, nuclear transfer lamb from cell clone PDCAAT90; lane 3, normal [illegible] lane 4, [illegible] and 8, non-targeted PDFF2 cells; lane 6 [illegible] lane 9, nuclear transfer lamb from clone PDCAAT90; lane 10, normal lamb; [illegible]

## Methods

### Gene-targeting vectors

The promoter-trap vector COLT-1 comprised a 3-kb region of the 3′ end of the ovine *COL1A1* gene from a point roughly 2.9-kb 5′ of the translation stop site to an *Ssp*I site 131-bp 3′ of the stop site; a 0.6-kb IRES region[18] corresponding to bases 1,247–1,856 of the pIREShyg vector (Clontech); a 1.7-kb region containing the bacterial *neomycin* gene and a portion of the 3′ end of the human growth hormone gene containing the polyadenylation site, essentially as described[24]; an 8.3-kb region of the 3′ end and flanking region of the ovine *COL1A1* gene from an *Ssp*I site 131 bp 3′ of the translational stop site to a *Xho*I site roughly 8.4-kb 3′ of the stop site; and the bacterial cloning vector pSL1180 (Pharmacia). DNA fragments homologous to the ovine *COL1A1* gene were derived from a single genomic clone isolated from a library of genomic fragments of PDFF2 in bacteriophage λ. The promoter trap transgene placement vector COLT-2 was constructed by inserting an *Mlu*I fragment containing the AATC2 transgene into COLT-1 at a unique *Eco*RV site at the 3′ end of the IRES–*neo* region.

### Preparation, culture and transfection of primary fibroblasts

Derivation of ovine PDFF2 and PDFF5 cells has been described[7]. PDFF cells were grown throughout in BHK 21 (Glasgow MEM) medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1X non-essential amino acids and 10% fetal calf serum in standard tissue-culture vessels in a humidified atmosphere composed of 2% $CO_2$, 5% $O_2$ and 93% $N_2$, and were passaged by standard trypsinization. PDFF cells were used at passage three and had undergone 5–6 days of culture following fetal disaggregation. Cells were plated at $5 \times 10^5$ cells in a 25-cm$^2$ flask and transfected the next day with either 6 μg of *Sal*I-linearized COLT-1 or *Sac*II-linearized COLT-2 using lipofectAMINE (Gibco, BRL Life Technologies) according to the manufacturer's guidelines. G418 selection (0.8 mg ml$^{-1}$) was applied 48 h after transfection. On average ~200 G418$^r$ colonies were derived per $5 \times 10^5$ cells transfected. Well-separated G418$^r$ colonies were isolated, expanded and cryopreserved by standard procedures.

### Nuclear transfer

Ovine cell clones were prepared for nuclear transfer by culture in medium containing reduced (0.5%) serum for either 2 or 4 days. Transfer of cell nuclei into Poll Dorset oocytes was carried out essentially as described[7].

### Nucleic acid analysis

Putative targeted cell clones were screened by PCR amplification across the 3′ short arm of homology. The position of PCR primers is shown in Fig. 1. Samples of cell clones for screening were lysed in PCR lysis buffer (50 mM KCl, 0.5 mM MgCl$_2$, 10 mM Tris HCl pH 8.5, 0.5% Nonidet P40, 0.5% Tween, 400 μg ml$^{-1}$ Proteinase K) at 65 °C for 30 min. Proteinase K was inactivated at 95 °C for 10 min, and PCR amplification was performed using the 'Expand long-template PCR system' (Boehringer) according to the manufacturer's recommended conditions. PCR primer sequences were: COLTPCR4 primer, 5′-GGATTGGTCCCAGGTGCTCA-3′; COLTPCR8 primer, 5′-GACCTTG-CATGCTTTTG CCCGAGAG-3′. Thermal cycling conditions were: 94 °C, 2 min; 10 cycles of 94 °C, 10 s, 55 °C, 30 s, 68 °C, 2 min; 20 cycles of 94 °C, 10 s, 60 °C, 30 s, 68 °C, 2 min + 20 s per cycle; followed by 68 °C, 7 min. PCR products were analysed by agarose gel electrophoresis.

Ovine genomic DNA was prepared from cell pellets, tail samples of live lambs and umbilical cord samples of dead lambs. Southern analysis was carried out by standard procedures. Three hybridization probes were used: a 5′ internal probe, a 3-kb *COL1A1* *Sall*–*Sspl* fragment corresponding to the 5′ homologous arm of COLT-1 and 2 (Fig. 1); a 5′ external probe, a 520-bp ovine *COL1A1* fragment directly adjacent to but outside the 5′ homologous arm; a 3′ internal probe, a 0.7-kb *COL1A1* *Sall*–*Pstl* fragment immediately 3′ of the integration site (Fig. 1). The diagnostic fragments detected were: a 4.2-kb *BamHI* fragment extending across the 3′ junction of the targeted locus from a *BamHI* site within the IRES–*neo* region to a *BamHI* site in the *COL1A1* gene 5′ of the region contained in the vector; a 8.4-kb *KpnI*–*AspI* fragment extending across the 3′ junction of the targeted locus from a *KpnI* site within the vector to an *AspI* site in the *COL1A1* gene flank 3′ of the region contained in the vector.

### Hormonal induction of lactation

Milk samples were obtained from immature ewes by hormonal induction of lactation, essentially as described[7].

Received 28 January; accepted 5 May 1999.


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### Acknowledgements

We would like to acknowledge the contributions of A. Gibson and K. Mrocek for embryo manipulation, L. Lynch and L. Hall of the Protein Biology technical assistance, T. Johnston for protein analysis and the PPU Operatives large animal team for animal husbandry and veterinary procedures. We thank L. Garner and D. Ayares for useful discussion.

[illegible] should be addressed to A.J.K.


# Gigantism in mice lacking suppressor of cytokine signalling-2


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Suppressor of cytokine signalling-2 (SOCS-2) is a member of the suppressor of cytokine signalling family, a group of related proteins implicated in the negative regulation of cytokine action through inhibition of the Janus kinase (JAK) signal transducers and activators of transcription (STAT) signal-transduction pathway[1]. Here we use mice unable to express SOCS-2 to examine its function *in vivo*. SOCS-2$^{-/-}$ mice grew significantly larger than their wild-type littermates. Increased body weight became evident after weaning and was associated with significantly increased long bone lengths and the proportionate enlargement of most organs. Characteristics of deregulated growth hormone and insulin-like growth factor-I (IGF-I) signalling, including decreased production of major urinary protein, increased local IGF-I production, and collagen accumulation in the dermis, were observed in SOCS-2-deficient mice, indicating that SOCS-2 may have an essential negative regulatory role in the growth hormone/IGF-I pathway.


We isolated genomic clones corresponding to three independent loci from two murine libraries using a SOCS-2 coding region complementary DNA as hybridization probe. Comparison of sequence from these clones with that of the SOCS-2 cDNA revealed that one locus, which consisted of three exons and two introns, encoded the predicted SOCS-2 RNA (Fig. 1a). The two other loci

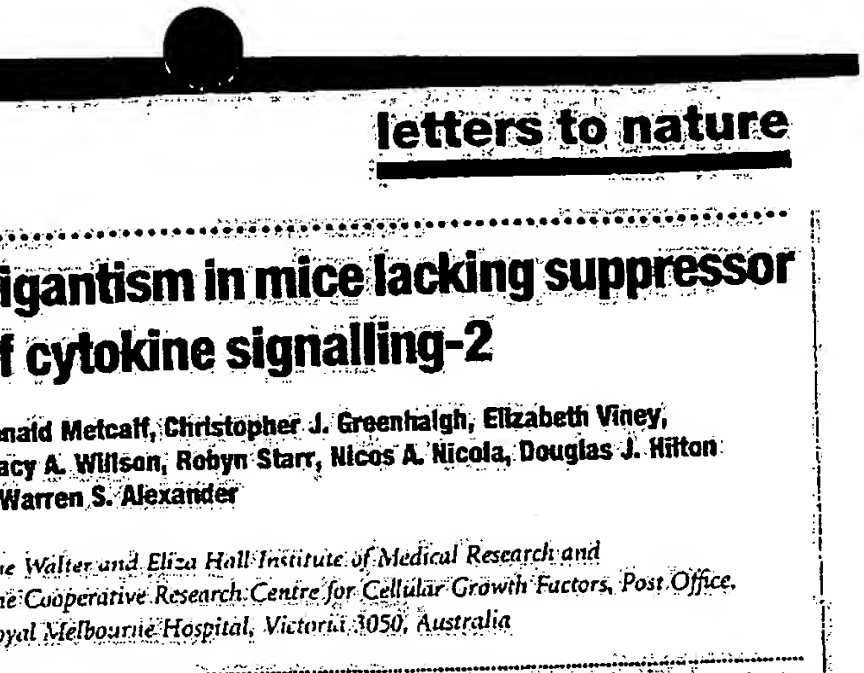


**Figure 1** Disruption of the SOCS-2 locus by homologous recombination. **a**, The functional murine SOCS-2 gene (B, *BamHI*; Nh, *NheI*; RV, *EcoRV*) with the exons containing the coding region as shaded boxes. In the targeted allele, the entire SOCS-2 coding region was replaced by a β-gal-PGKneo cassette in which the β-galactosidase coding region was fused to the SOCS-2 initiation codon. **b**, Southern blot of *EcoRV*-digested genomic DNA from the tails of mice derived from a cross between SOCS-2$^{+/-}$ mice. The blot was hybridized with the 5′ genomic SOCS-2 probe, which distinguishes between endogenous (16 kb) and mutant SOCS-2 (9 kb) alleles. **c**, Northern blot showing lack of SOCS-2 expression in organs of SOCS-2$^{-/-}$ mice. Top, the blot was hybridized with a coding region probe, which detects the 3.4 kb SOCS-2 transcript; bottom, the integrity of the RNA was confirmed by hybridization with GAPDH (1.4 kb transcript). Spl gl, salivary gland.

data demonstrate that compound 4 is a potent LFA-1 antagonist, which binds LFA-1, blocks the binding of ICAM-1, and inhibits LFA-1 mediated lymphocyte proliferation and adhesion in vitro. It achieves this with a potency significantly greater than cyclosporine A, and demonstrates its equivalence to anti-CD11a in the level of its inhibition of the immune system's response in vivo.

Compounds 1 through 4 emerged from considerations of the ICAM-1 epitope via kistrin, the RGDMP peptides and $H_2N$-CGY$^{(m)}$DMPC-COOH. Each of these LFA-1 ligands was able to compete with a fluorescein-conjugated analog of compound 3 for LFA-1 binding. SAR similarities suggest a common presention of a carboxylic acid moiety to a binding site on LFA-1 as the basis of this competition. A comparison of the structures and molecular functionality of compound 4 and ICAM-1 responsible for their LFA-1 binding reveals that the carboxylic acid, sulfide, phenol, and carboxamide groups of the ICAM-1 epitope are embodied in compound 4 (Fig. 3) (*14*). This allows us to propose that compounds 2 through 4 are mimics of ICAM-1 resulting from the transfer of the ICAM epitope to a small molecule. A definitive proof of this mimicry will require the determination of the structures of LFA-1 and its complexes with ICAM-1 and compounds 2 through 4.

We believe the work presented here (*23*, *24*) represents the first reduction of a nonlinear, discontinuous but contiguous protein epitope (encompassing five residues spanning three different β strands across the face of a protein surface) from a protein to a small molecule. In contrast to more traditional approaches, this rational, structurally directed hypothesis and information driven lead discovery process utilized molecular modeling and structure activity relationships to identify pharmacophoric similarities within ICAM-1, kistrin, the peptides, and ultimately compounds 1 through 4. This provided the perspective to recognize compound 1 as a viable lead and rapidly elaborate it into compounds 2 through 4, and demonstrates the value of antibodies, protein mutagenesis, and structural (SAR) data for native protein ligands as leads in the identification of pharmaceutical agents, which block large protein-protein interactions. What remains to be seen with these small-molecule LFA-1 antagonists is a clinical evaluation of their safety and effectiveness in the control of human diseases relative to humanized anti-CD11a and other small-molecule agents discovered by other means (*25*–*28*).

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24. In the course of the studies presented here, several reports appeared describing small molecule antagonists of LFA-1/ICAM (*25*–*27*). Two have identified compounds which interact with LFA-1's allosteric I domain site (*26*, *27*). Preliminary studies with compound 4 indicate that it binds at a different site on LFA-1, and that the allosteric site antagonist(s) do not inhibit the binding of compound 4 to LFA-1.
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30. We thank P. Barker, C. Quan, J. Tom, and M. Struble for help with peptide synthesis and compound purification, S. Spencer for protein preparation and purification, and J. Burnier, R. Lazarus, and R. G. Hammonds for helpful discussions in the preparation of the manuscript.

17 September 2001; accepted 20 December 2001

# Production of α-1,3-Galactosyltransferase Knockout Pigs by Nuclear Transfer Cloning

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The presence of galactose α-1,3-galactose residues on the surface of pig cells is a major obstacle to successful xenotransplantation. Here, we report the production of four live pigs in which one allele of the α-1,3-galactosyltransferase locus has been knocked out. These pigs were produced by nuclear transfer technology; clonal fetal fibroblast cell lines were used as nuclear donors for embryos reconstructed with enucleated pig oocytes.

Clinical transplantation has become one of the preferred treatments for end-stage organ failure since the introduction of chronic immunosuppressive drugs in the mid-1980s. One of the novel approaches to dealing with the limited supply of human organs is the use of alternative species as a source of organs (xenotransplantation). The pig is considered the primary alternative species because of ethical considerations, breeding characteristics, infectious disease concerns, and its compatible size and physiology (*1*).

A major barrier to progress in pig-to-primate organ transplantation is the presence of terminal α-1,3-galactosyl (Gal) epitopes on the surface of pig cells. Humans and Old World monkeys have lost the corresponding galactosyltransferase activity in the course of

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evolution and therefore produce preformed natural antibodies to the epitope, which are responsible for hyperacute rejection of porcine organs. The temporary removal of recipient antibodies to Gal through affinity adsorption and expression of complement regulators in transgenic pigs has allowed survival of pig organs beyond the hyperacute stage. However, returning antibody and residual complement activity are believed to be responsible for the acute and delayed damage that severely limit organ survival even in the presence of high levels of immunosuppressive drugs and other clinical intervention (*2*). Competitive inhibition of galactosyltransferase in α-1,2-fucosyl-transferase transgenic pigs has resulted in only partial reduction in epitope numbers (*3*). Similarly, attempts to block expression of Gal epitopes in *N*-acetylglucosaminyltransferase III transgenic pigs also resulted in partial reduction of Gal epitope number but failed to substantially extend graft survival in primate recipients (*4*). Given the large number of Gal epitopes present on pig cells (*5*), it seems unlikely that any dominant transgenic approach of this nature can provide sufficient protection from damage mediated by antibodies to Gal. In contrast, a genetic knockout of the α-1,3-galactosyltransferase (GGTA1) locus in pigs would provide permanent and complete protection.

Viable α-1,3-galactosyltransferase knockout mice have been produced by embryonic stem cell technology (*6*). The development of nuclear transfer technology has provided a means for locus-specific modification of large animals, as demonstrated by the production of viable sheep by means of in vitro targeted somatic cells (*7*). Successful cloning (*8*–*11*) and production of transgenic pigs by nuclear transfer of genetically modified somatic cells (*12*) have been reported. Attempts at targeting the GGTA1 locus in pigs (*11*) and sheep (*13*) have also been reported, but these failed to result in live birth of animals with the desired modification. In both cases, difficulties in obtaining viable targeted donor cell clones were encountered.

We chose to knock out the GGTA1 locus in a highly inbred, major histocompatibility complex–defined miniature pig line. Descendant from lines long used for xenotransplantation studies (*14*, *15*), this line is an ideal size match for eventual use in clinical transplantation and has animals that consistently test negative for transmission of porcine endogenous retrovirus (PERV) to human cells in vitro (*16*). Cells were isolated from one male (F9) and three female (F3, F6, and F7) fetuses at day 37 of gestation for production of donor cell lines (*17*). A gene trap targeting vector, pGalGT, was used for homologous replacement of an endogenous GGTA1 allele (Fig. 1). The vector contains about 21 kb of homology to the GGTA1 locus, with the coding region upstream of the catalytic domain disrupted by insertion of a selection cassette consisting of a Bip internal ribosome entry site followed by sequences encoding G418 resistance. After transfection and 14 days of G418 selection, viable cell clones (*18*) were passaged in triplicate for further analysis and cryopreservation (*19*).

A reverse transcription polymerase chain reaction (RT-PCR) was performed on crude cell lysates the day after passage with a forward primer from exon 7 (upstream of the 5′ end of the targeting vector) and a reverse primer from the selection cassette (*20*). Dot blot hybridization of the RT-PCR products with an exon 8 probe detected targeting in 22 of 159 clones analyzed.

The structure of the GGTA1 locus was analyzed in two overlapping PCR reactions (*21*). Clones with a targeted insertion of the cassette relative to vector external primer sites both upstream and downstream of the cassette, indicative of a replacement-type targeting event, were considered candidates for use in nuclear transfer. Of 17 clones analyzed, 8 were found to have undergone the desired recombination event, and one from each fetus (F3-C5, F6-C3, F7-H6, and F9-J7) was used for nuclear transfer.

Nuclear transfer was performed with the use of in vitro matured oocytes and, except for 4 of 28 embryo transfers, cryopreserved donor cells without further culture (*22*). Asynchronous embryo transfer—that is, transfer to a surrogate at an earlier stage of the estrus cycle than the embryos themselves—had previously been used with minimally manipulated (*23*), pronuclear microinjected (*24*), and nuclear transfer (NT)–derived embryos (*10*–*12*). The observed benefit of asynchronous transfer suggests that any manipulation may result in a delay in early embryonic development. Because the manipulations required for nuclear transfer are quite extensive, and because previous reports suggest that miniature swine embryos of the NIH strain used here may normally develop at a relatively slower rate (*25*), naturally cycling large white gilts that had displayed standing estrus but had not yet completed ovulation were used as surrogates (*26*). [For detailed information on all 28 embryo transfers, see (*18*).]

A minimum of four viable embryos is required for establishment of pregnancy in pigs (*27*). Thus, we used two methods to increase the likelihood of establishing pregnancies with NT-derived embryos. Although pregnancy was established in five of seven surrogates receiving parthenogenetic "carrier" embryos, no live births resulted. Therefore, we transferred reconstructed embryos to a mated surrogate. In the one embryo transfer performed in this group, the surrogate (O212) was mated on the first day of standing estrus and received NT-derived embryos the same day. Although any fertilized embryos would theoretically be 43 to 55 hours behind development of the transferred NT-derived embryos, the actual in vivo development rate for NT-derived embryos is unknown. Early pig embryos have a lower rate of survival when present in a surrogate along with embryos at a slightly more advanced stage (*25*). Thus, an apparent embryonic asynchrony may be advantageous should NT-derived embryos develop at a slower rate than naturally fertilized embryos.

Seven piglets, four females and three males (Table 1), were delivered by cesarean section at term (*28*). Microsatellite analysis (*29*) revealed that six of six haplotypes for one female piglet (O212-2) were iden-

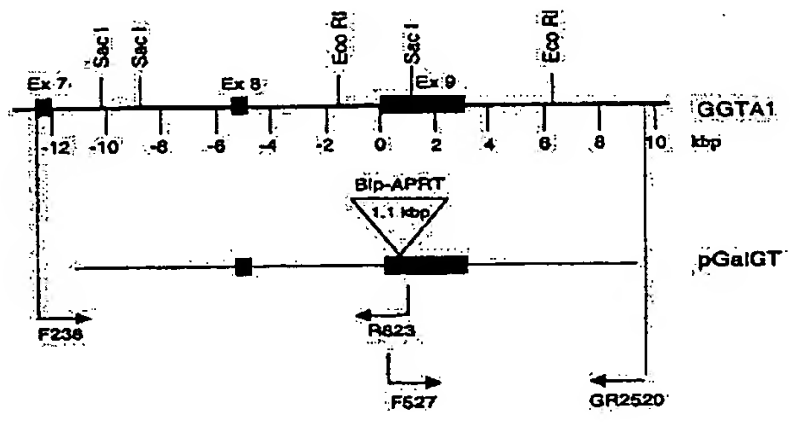


Fig. 1. pGalGT targeting vector and genomic PCR assays for targeting (*35*).

Table 1. Pregnancies carried to term after transfer of embryos reconstructed with GGTA1 knockout cell lines.

| Surrogate (estrus day) | Donor line | NT embryos | Outcome |
|---|---|---|---|
| O212 (0) | F7-H6 | 116 | Mated surrogate<br>Seven born 9/21/01<br>One NT-derived female piglet |
| O226 (1) | F3-C5 | 92 | Four NT-derived female piglets born 10/19/01 |
| O230 (1) | F7-H6 cultured | 130 | Two NT-derived female piglets born 10/15/01 |

tical to that of the F7 fetal cell line from which knockout donor line F7-H6 was derived (*18*). Furthermore, three of six haplotypes of O212-2 were not compatible with mating of the surrogate. All other piglets had at least four haplotype mismatches with the F7 line and were compatible with mating of the surrogate.

We performed 20 additional transfers of only NT-derived embryos to unmated surrogates. Pregnancy was confirmed by ultrasound in six of these surrogates, with two continuing to cesarean section at term. Two live piglets were delivered from surrogate O230 (Table 1), one of which (O230-2) died from respiratory distress syndrome shortly after delivery. Four live piglets were delivered from surrogate O226 (Table 1), again with one (O226-4) dying shortly after birth from respiratory distress syndrome. Microsatellite haplotypes of all six piglets from the two litters were identical to the F7 and F3 donor parental cell lines, respectively.

Genomic targeting analysis was performed on DNA samples from all NT-derived piglets, the untransfected F3 and F7 donor parental cell lines, and surrogates (Fig. 2). For all piglets except O230-2, analysis of both ends of the GGTA1 locus revealed the presence of one replacement-type targeted allele. Whether O230-2 was derived from an untargeted miniature swine cell in the F7-H6 donor line or had a GGTA1 rearrangement incompatible with detection by the targeting assays performed has yet to be determined.

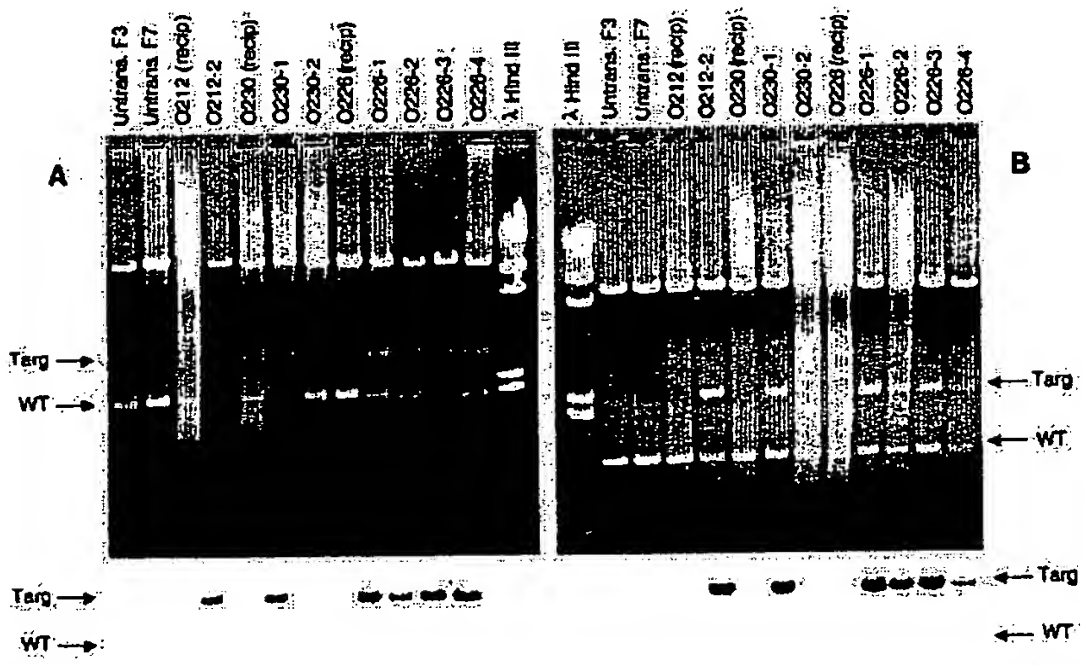


Fig. 2. Targeting analysis of NT-derived piglets, parental miniature swine fetal cell lines F3 and F7, and surrogate sows. See Fig. 1 for a description of the assays. (A) Upstream genomic PCR analysis with primers F238 and R823. (B) Downstream genomic PCR analysis with primers F527 and GR2520. After transfer, digested reactions were probed with an oligonucleotide (Bip419) from the IRES portion of the selection cassette. The analysis of all offspring, with the exception of O230-2, is consistent with a replacement-type targeting event at one GGTA1 allele.

Table 2 presents a health summary for the seven NT-derived piglets. Four of the five piglets surviving beyond the immediate postpartum period remain healthy, with a normal growth rate for miniature swine. The fifth, O226-3, died suddenly at 17 days of age during a routine blood draw. Necropsy revealed a dilated right ventricle and thickening of the heart wall. Another animal, O230-1, has shown cardiac defects similar to those reported in NT-derived animals of other species (*7*, *12*, *30*, *31*).

A number of other abnormalities were noted at birth among surviving piglets, none of which appear to affect their overall health and well-being. Flexure tendon deformities similar to those reported here have been observed in previous NT-derived commercial strain pigs (*32*). It is unlikely that the abnormalities we have seen are related to the genetic modification, as there is not a consistent phenotype and only one allele has been targeted, but rather are the result of improperly reprogrammed epigenetic factors. With the exception of O212-2, the four surviving piglets were somewhat undersized, with birth weights of 450 to 650 g (strain average 860 g).

Under our growth and selection conditions, miniature swine fetal fibroblasts maintain a steady doubling time of about 24 hours. Clonal lines senesce after 30 to 32 days of culture on average. The ability to quickly select clonal lines for nuclear transfer is likely to be a requirement for introduction of other complex genetic alterations into the pig genome. The ability to use cryopreserved donor cells without further culture, demonstrated by two of our litters, is also advantageous, as it extends the number of potential donor lines available for use in nuclear transfer. Our efficiencies in producing NT-derived GGTA1 knockout animals are similar to those previously reported in which extensively cultured primary fetal cells were used as

Table 2. Health summary of NT-derived miniature swine piglets. H, healthy; NG, normal growth; D, dead.

| Piglet | Physical findings | Birth weight (g) | Clinical symptoms/cardiac exam findings |
|---|---|---|---|
| O212-2 (H, NG) | Ocular defect, small ear flaps with no patent ear canals | 1100 | No clinical symptoms; no significant echocardiogram findings |
| O230-1 (H, NG) | Flexure deformity of distal interphalangeal joint at birth* | 450 | Mild abdominal ascites; right ventricular enlargement and pulmonary hypertension |
| O230-2 (D) | Flexure deformity of distal interphalangeal joint at birth; dysmaturity at birth | 115 | Died shortly after delivery of respiratory distress syndrome; no gross lesions observed at necropsy |
| O226-1 (H, NG) | Normal | 600 | No clinical symptoms; no significant echocardiogram findings |
| O226-2 (H, NG) | Flexure deformity of distal interphalangeal joint at birth† | 650 | No clinical symptoms; low-velocity regurgitation at center of tricuspid valve |
| O226-3 (D) | Flexure deformity of distal interphalangeal joint at birth* | 550 | Death during routine blood draw 17 days after birth; dilated right ventricle with thickening of heart wall observed at necropsy |
| O226-4 (D) | Cleft palate; dysmaturity at birth | 250 | Died shortly after delivery of respiratory distress syndrome; no gross lesions observed at necropsy |

*Responded to physical therapy. †Responded to physical therapy plus splinting.

nuclear donors (*10*, *11*), despite the nearly fourfold difference in adult size between the miniature swine strain modified here and the commercial oocyte donor and surrogate strains used. The ability to use readily available oocyte donors and surrogates in a nuclear transfer program is essential when modification of less commonly available animals is required.

The next step will be to create α-1,3-galactosyltransferase–null (homozygous knockout) pigs, either by breeding to a heterozygous male produced by nuclear transfer or by sequential nuclear transfer modification of cell lines produced from the four female pigs reported here. Because α-1,3-galactosyltransferase–null mice have already been produced (*6*), it is not anticipated that this genetic modification will be lethal in the null animals. We hope that α-1,3-galactosyltransferase–null pigs will not only eliminate hyperacute rejection but also ameliorate later rejection processes, and (in conjunction with clinically relevant immunosuppressive therapy) will permit long-term survival of transplanted porcine organs. At a minimum, the availability of galactosyltransferase-null pigs will allow a clearer evaluation of approaches currently in development aimed at overcoming potential delayed and chronic rejection mechanisms in porcine xenotransplantation.

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15. Fetuses for derivation of cell lines used here were the product of six generations of brother-sister mating and have an estimated inbreeding coefficient of 0.86. Two additional sib-mated generations have been produced from this inbred line, with no apparent decrease in fitness.
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17. Primary fibroblasts were isolated from miniature swine fetuses by collagenase-trypsin digestion of minced tissue. Dissociated cells were plated at 2 × $10^5$ cells/$cm^2$ on collagen-coated plates in Ham's F10 medium containing 20% fetal bovine serum (FBS) and antibiotics. Adherent cells were frozen the following day.
18. For supplementary data, see *Science* Online (www.sciencemag.org/cgi/content/full/1068228/DC1).
19. Fetal fibroblasts were thawed and cultured for 3 days to subconfluence before transfection. About 2 × $10^7$ fibroblasts were electroporated at 260 V, 960 μFD in 0.8 ml of Hepes-buffered saline containing pCalGT (0.5 pmol/ml). The vector was restriction-digested at both ends of the GATA1 homologous sequences before use. Transfected cells were cultured in bulk for 2 days without selection, then plated in collagen-coated 96-well plates at 2 × $10^4$ cells per well in Ham's nutrient mixture F10 containing 20% FBS and G418 (100 μg/ml). The low selection concentration was made possible by the absence of cells transiently expressing G418 resistance; untransfected cells were uniformly killed by G418 (50 to 75 μg/ml) after 5 to 7 days. After 14 days of selection in G418, growing cultures were passaged in triplicate for cryopreservation of donor cells, RT-PCR screening for targeting, and DNA isolation. Subconfluent donor cell cultures were trypsinized and frozen in 20-μl aliquots containing 1000 to 2000 cells each.
20. Lysates were prepared from 96-well cultures of selected clones the day after passage by three rounds of freezing and thawing in 10 μl of 2 mM dithiothreitol containing placental ribonuclease inhibitor (1 U/μl). The lysate was amplified in a one-tube amplification reaction using rTth polymerase, exon 7 forward primer F291 (5′-ACAACAGAGGAGAGCTTCCG) and reverse primer Bip419 (5′-CTCTCACACTCGCGAAACAC). PCR products were alkaline-denatured and transferred to nylon membranes before hybridization with an exon 8 oligonucleotide probe (5′-GGTCGTGACCATAACCAGATG).
21. Clones identified as putatively targeted in the RT-PCR screening assay were expanded into 24-well plates, and DNA was isolated for genomic analysis. About 250 ng of DNA was amplified in reactions with LA Taq DNA polymerase (Panvera, Madison, WI). Targeting was assessed by two PCR assays, each incorporating a primer outside of the vector homologous region. Upstream analysis used exon 7 forward primer F238 (5′-TTACCACGAAGAAGAAGACGC) and exon 9 reverse primer R823 (5′-AGGATGTGCCTTGTACCACC). Upon digestion with Eco RI, fragments of 2.0 kb (WT locus), 3.1 kb (targeted locus), and 10.4 kb (either locus) are produced. Downstream analysis used exon 9 forward primer F527 (5′-GGTTGGCCACAAAGTCATC) and reverse primer GRZ520 (5′-CACTATTTGGAGGACAGCGTC). Upon digestion with Sac I, fragments of 1.2 kb (WT locus), 2.3 kb (targeted locus), and 8.1 kb (either locus) are produced. Southern blots of digested reactions were hybridized to internal ribosome entry site (IRES) region probe Bip419.
22. Oocytes derived from slaughtered gilts were matured in defined protein medium [TCM 199 supplemented with 0.1% polyvinyl alcohol, cysteine (0.1 mg/ml), epidermal growth factor (10 ng/ml), 0.91 mM Na-pyruvate, 3.05 mM D-glucose, follicle-stimulating hormone (0.5 μg/ml), luteinizing hormone (0.5 μg/ml), penicillin (75 μg/ml), and streptomycin (50 μg/ml)]. Oocytes from sow ovaries were purchased from BoMed Inc. (Madison, WI) and shipped overnight in their commercial maturation medium. After maturation, oocytes were freed of cumulus cells and were kept in TCM/BSA [TCM 199 supplemented with bovine serum albumin (BSA, 4 mg/ml)] until use. Enucleation of metaphase II oocytes was performed in medium supplemented with cytochalasin B (7.5 μg/ml), without staining the chromatin (as this may be detrimental to subsequent development) (*33*). Cryopreserved donor cells were thawed at 37°C and 10 volumes of FBS were added. The suspension was kept at room temperature for 30 min, and four volumes of TCM/BSA were added and the cells pelleted. Fibroblast cells were resuspended and directly used for NT. For NT-derived embryos transferred to four surrogates (O230, O203, O291, and O221), the cells were cultured for 1 week as above, then overnight in medium containing 0.5% serum before use in NT. All cells with an intact membrane were used, as the limited number of targeted cells did not permit selection. Nuclear transfer, fusion, and activation were performed as in Park *et al.* (*12*). Embryos were kept in TCM/BSA for another 30 to 60 min before the fusion rate was evaluated. Fused embryos were cultured in NCSU 23 supplemented with BSA (4 mg/ml) overlaid with mineral oil. The surviving embryos (intact plasma membrane) were selected for transfer into surrogates.
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26. Potential surrogates were checked for estrus twice a day. Depending on the exact time of estrus, NT-derived embryo transfers were performed 5 to 17 hours or 20 to 36 hours after the actual onset of estrus for day 0 and day 1 surrogates, respectively. In prior control experiments using in vitro produced embryos cultured for 22 hours after fertilization and then transferred to a day 1 surrogate, 19 of 100 embryos recovered on day 6 were at blastocyst stage, with an average nuclear number of 65. For surgery, gilts were induced with Pentothal (Abbott Laboratories) and anesthesia was maintained with 2% Halothane (Halocarbon Laboratories, River Edge, NJ). A midventral laparotomy was performed, and embryos were loaded into a 3½ Fr. tomcat catheter and deposited into the oviduct. Examination of the ovaries during embryo transfer confirmed that none of the surrogates had completed ovulation.
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28. Surrogates displayed few if any signs of impending parturition, similar to findings reported in other species (*31*, *34*). They also lacked mammary gland development and colostrum production. The surrogates all displayed mild signs of vulvar enlargement and elongation, but much less than would normally be expected. This would appear to indicate a defect in the signaling mechanism between the fetuses and dam, and would most likely result from an abnormality in placental function. These observations were made even in the mated recipient.
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35. GGTA1 homologous sequences in the pGalGT vector begin ~0.8 kb downstream of exon 7 and continue to ~6.8 kb downstream of the end of exon 9. A selection cassette—consisting of a Bip internal ribosome entry site, APRT coding sequences (encoding G418 resistance), and flanking stop codons—is inserted into an Eco RV site upstream of the GGTA1 catalytic domain in exon 9. Targeting is assessed with two PCR assays, each incorporating a primer outside of the vector homologous region. Upstream genomic structure is assessed with primers F238 (exon 7, upstream of the 5′ end of the pGalGT vector) and R823 (exon 9 downstream of the selection cassette insertion site). Upon digestion with Eco RI, fragments of 2.0 kb (WT locus), 3.1 kb (targeted locus), and 10.4 kb (either locus) are produced. Downstream genomic structure is assessed with primers F527 (exon 9 upstream of the selection cassette insertion site) and GR2520 (downstream of the 3′ end of the pGalGT vector). Upon digestion with Sac I, fragments of 1.2 kb (WT locus), 2.3 kb (targeted locus), and 8.1 kb (either locus) are produced.



36. We thank E. Brown and D. Liske for care of the surrogate gilts during gestation; T. Cantley for help with surgical embryo transfers; J. C. Lattimer for echocardiography; K. Whitworth, R. Woods, J. Luth, L. Overman, R. Cabot, and D. Wax for helping to care for the piglets after delivery; S. Am for miniature swine management and assistance; M. Baetscher and D. Akiyoshi for their contributions to the early phases of cell engineering work; and C. Patience for comments on the manuscript. Supported by the F. B. Miller fund, Department of Animal Sciences, University of Missouri–Columbia (H.-T.C.), NIH grant T32 RR07004 (D.B.C.), and NIH National Center for Research Resources grant R44 RR15198.

20 November 2001; accepted 17 December 2001
Published online 3 January 2002;
10.1126/science.1068228
Include this information when citing this paper.

# Targeted disruption of the α1,3-galactosyltransferase gene in cloned pigs

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Galactose-α1,3-galactose (α1,3Gal) is the major xenoantigen causing hyperacute rejection in pig-to-human xenotransplantation. Disruption of the gene encoding pig α1,3-galactosyltransferase (α1,3GT) by homologous recombination is a means to completely remove the α1,3Gal epitopes from xenografts. Here we report the disruption of one allele of the pig α1,3GT gene in both male and female porcine primary fetal fibroblasts. Targeting was confirmed in 17 colonies by Southern blot analysis, and 7 of them were used for nuclear transfer. Using cells from one colony, we produced six cloned female piglets, of which five were of normal weight and apparently healthy. Southern blot analysis confirmed that these five piglets contain one disrupted pig α1,3GT allele.

Galactose-α1,3-galactose (α1,3Gal) epitopes are a common carbohydrate structure on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys[1]. Synthesis of the α1,3Gal epitope is catalyzed by the enzyme α(1,3)galactosyltransferase (α1,3GT)[2]. Humans do not have a functional copy of the α1,3GT gene, and hence do not show α1,3Gal surface expression. The presence of the α1,3Gal antigen on the surface of pig cells and tissues is the major cause of hyperacute rejection (HAR) in pig-to-human xenotransplantation[2–4]. It has been reported that, in humans, up to 1% of the total circulating IgG is anti-α1,3Gal natural antibody[5]. A number of strategies have been used to reduce or eliminate α1,3Gal-induced HAR. These methods[2–4] include overexpression of α2,3-sialyltransferase or α1,2-fucosyltransferase in pig cells to compete with α1,3GT; treatment of pig organs with α-galactosidase to remove surface α1,3Gal epitopes; expression of complement inhibitor genes, such as human decay-accelerating factor (DAF), in transgenic pig organs to suppress the complement reaction; and temporary depletion of natural anti-α1,3Gal antibody from recipients before transplantation. All these methods only partially or temporarily remove the α1,3Gal from the surface of the xenografts, however, and the residual α1,3Gal molecules are still sufficient to activate the complement cascade and cause destruction of the grafts[2–4]. Complete elimination of α1,3Gal epitopes from the donor organs should be achievable by removal of the α1,3GT gene. α1,3GT knockout mice have been made by a number of groups[6–7]. When tissues from these mice are exposed to human serum, they bind substantially less human anti-Gal xenoantibody than do tissues from normal mice, resulting in a significant decrease in human complement activation[6].

The cloning of sheep[8], goat[9], cattle[10], and pigs[11] by somatic cell nuclear transfer provides an alternative means of disrupting or deleting genes in mammals other than mice. The production of cloned sheep with targeted insertions at the ovine α1(I)-procollagen (*COL1A1*) locus showed that viable animals can be produced via nuclear transfer with gene-targeted cultured fibroblasts[12]. The α1,3GT gene has recently been successfully deleted in sheep fibroblasts and in fetuses cloned from targeted cells[13]. Although no viable animals resulted, these experiments showed that it is feasible to disrupt the α1,3GT gene using nuclear transfer techniques in livestock. Lai *et al.* have recently described the disruption of one allele of the α1,3GT gene in pig fibroblasts and in four live piglets cloned from these cells[14]. However, the only evidence of gene targeting offered in this report was PCR analysis of recombination junctions. Here we present genomic Southern blot analyses showing successful disruption of one copy of the α1,3GT gene in cultured male and female porcine fetal fibroblasts. To date we have produced five apparently healthy α1,3GT knockout female piglets by nuclear transfer.

## Results and discussion

Because the α1,3GT gene is expressed well in porcine fetal fibroblasts (PPL Therapeutics, unpublished data), it is possible to enrich for homologous recombination events using a promoter-trap knockout-vector strategy[12]. Two similar knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of SLA1-10 and PCFF4-2 cells, respectively, by inserting an *IRES-neo*-poly A cassette into the 5′ end of exon 9 (Fig. 1A). Because the majority of the coding region of the pig α1,3GT gene, including the sequences encoding the catalytic domain, is located in exon 9, successful targeting using these vectors is expected to result in functional inactivation of the gene[6–7].

Four different early-passage (P2 or P3) primary porcine fetal fibroblasts cell lines were used for transfection: the male cell lines SLA1-10, PCFF4-2, and PCFF4-3 and the female cell line PCFF4-6. SLA1-10 cells were transfected with the isogenic vector pPL654, and PCFF4-2 cells were transfected with the isogenic vector pPL657. The PCFF4-3 and PCFF4-6 cell lines were derived from sibling fetuses of the fetus used to derive PCFF4-2, and therefore were transfected with the pPL657 vector. G418-resistant colonies were screened by 3′ PCR with neo442S (a sequence from the 3′ end of *neo*) and αGTE9A2 (a sequence from the 3′ end of exon 9 in sequences located outside the 3′ recombination

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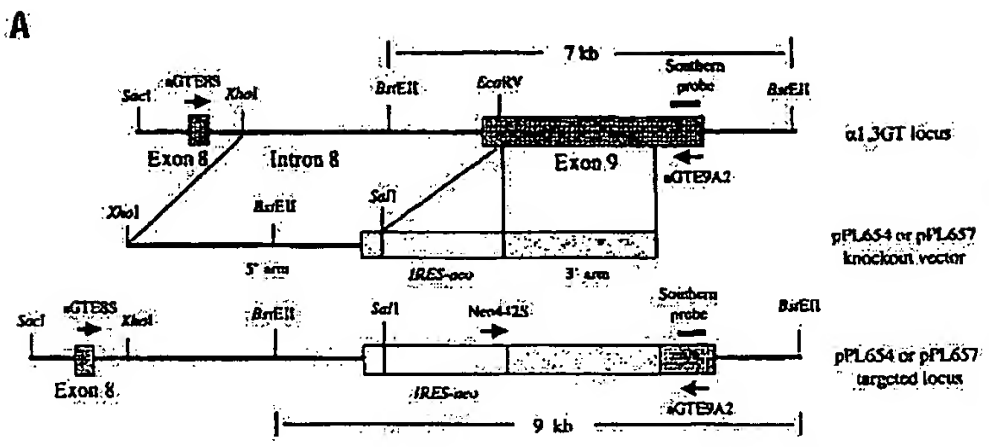


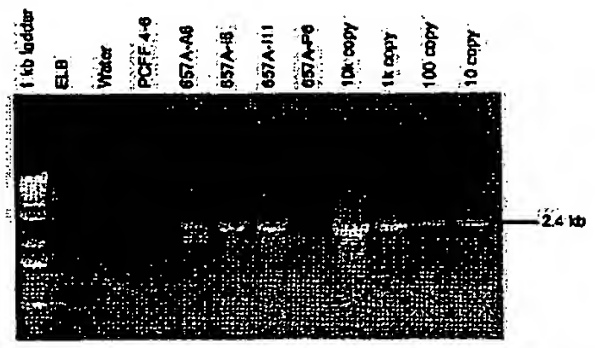


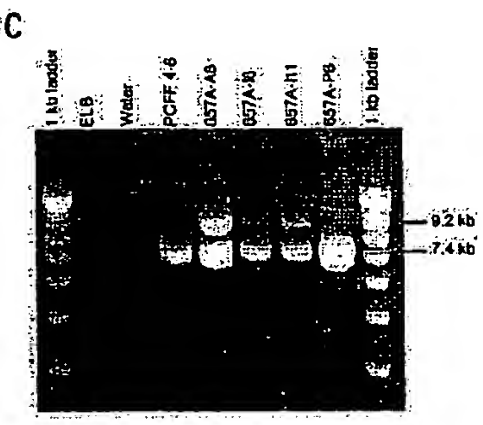


**Figure 1.** Disruption of the porcine α1,3GT gene by gene targeting. (A) Diagram of the porcine α1,3GT locus, the corresponding α1,3GT genomic sequences used as 5′ and 3′ arms in the α1,3GT knockout vectors, and the structure of the targeted locus after homologous recombination. The two α1,3GT knockout vectors (pPL654 and pPL657) have the same design except that the α1,3GT sequences present in the recombination arms are derived from different cells. The names and positions of the primers used for 3′ PCR and long-range PCR are indicated by short arrows: Neo442S: 5′-CATCGCCTTCTATCGCCTTCTT-3′; αGTE8S: 5′-AGAGGTCGTGACCATAACCAGAT-3′; αGTE9A2: 5′-AGCCCATCGTGCTGAACATCAAGTC-3′. The short bar indicates the probe used for α1,3GT Southern blot analysis. The predicted size of Southern hybridization bands with *Bst*EII digestion, for both the endogenous α1,3GT locus and the α1,3GT targeted locus, is indicated. (B) 3′ PCR results. Approximately 1,000 cells were used for 3′ PCR, with Neo442S as the forward primer and αGTE9A2 as the reverse primer. Three negative controls, embryo lysis buffer (ELB), water, and wild-type (PCFF4-6) cells, were used to ensure that the PCR reaction was not contaminated. 657A-A8, 657A-I6, 657A-I11, and 657A-P6 are individual G418-resistant colonies. A positive-control plasmid was included, at dilutions from 10,000 copies down to 10 copies as indicated. (C) LR-PCR results. Approximately 1,000 cells were used, with αGTE8S as the forward primer and αGTE9A2 as the reverse primer. ELB, water, and pCFF4-6 cells were used as negative controls. 657A-A8, 657A-I6, 657A-I11, and 657A-P6 are individual G418-resistant colonies.

arm) as forward and reverse primers (Fig. 1A). Thus, only through successful targeting at the α1,3GT locus would the expected 2.4-kb PCR product be obtained. From a total of seven transfections in four different cell lines, 1,105 G418-resistant colonies were picked, of which 100 (9%) were positive for α1,3GT gene disruption in the initial 3′ PCR screen (range 2.5–12%; Table 1). Figure 1B shows the 3′ PCR results for a representative group of G418-resistant colonies. Colonies 657A-A8, 657A-I6, and 657A-I11 showed the expected 2.4-kb band, whereas control PCFF4-6 cells and another G418-resistant colony, 657A-P6, did not. A portion of each 3′ PCR–positive colony was frozen immediately in several small aliquots for future use in nuclear transfer experiments, and the rest of the cells were expanded for long-range PCR (LR-PCR) and Southern blot analysis.

From our and others experience[15-16], we expected that DNA analysis by PCR, or mRNA analysis by reverse transcription PCR, to detect recombination junctions would be prone to generating false-positive results. Therefore, to further confirm successful targeting at the α1,3GT locus, we carried out an LR-PCR experiment encompassing the entire targeted region. The LR-PCR covered the 7.4-kb α1,3GT genomic sequence from exon 8 to the end of exon 9, with both primers (αGTE8S and αGTE9A2) located outside the recombination region (Fig. 1A). The control PCFF4-6 cells and the 3′ PCR–negative colony, 657A-P6, showed only the endogenous 7.4-kb band from the wild-type α1,3GT locus (Fig. 1C). In contrast, three of the 3′ PCR–positive colonies, 657A-A8, 657A-I6, and 657A-I11, showed both the 7.4-kb endogenous band and a new band of 9.2 kb, the size expected for targeted insertion of the 1.8-kb *IRES-neo* cassette into the α1,3GT locus. As some 3′ PCR–positive signals may come from PCR artifacts, the LR-PCR assay is crucial to confirm successful knockout events. As evidence of this fact, only 30% of the 3′ PCR–positive colonies could be confirmed by LR-PCR (Table 1).

Approximately half (17/30) of the LR-PCR–positive colonies were successfully expanded to yield enough cells ($1 \times 10^6$) for Southern blot analysis. We expected that the colonies would be heterozygous for knockout at the α1,3GT locus and thus would have one normal copy and one disrupted copy of the α1,3GT gene. With *Bst*EII digestion, the α1,3GT knockout cells should show two bands: a 7-kb band of the size expected for the endogenous α1,3GT allele and a 9-kb band characteristic of insertion of the *IRES-neo* sequences at the α1,3GT locus (Figs. 1A, 2). Southern blot analysis confirmed knockout of the gene in all 17 LR-PCR–positive colonies. The same membranes were re-probed with sequences specific for *neo*, and the 9-kb band was detected with the *neo* probe (data not shown), confirming the targeted insertion of the *IRES-neo* cassette at the disrupted α1,3GT locus. Table 1 summarizes the results of transfection, 3′ PCR, LR-PCR, and Southern blot analysis. Recombination frequencies were highest in the PCFF4-2 and PCFF4-6 cell lines. On the basis of the LR-PCR results, the overall α1,3GT knockout rate in G418-resistant colonies was 6% for PCFF4-2 cells, 3% for PCFF4-6 cells, and 0.5% for PCFF4-3 cells. The fact that PCFF4-2 cells gave the highest recombination frequency may be related to isogenicity

**Table 1. Summary of 3′ PCR, LR-PCR, and Southern analysis results of G418-resistant colonies**

| Cells (sex) | Knockout vectors | No. of G418$^R$ colonies | No. of 3′ PCR$^+$ colonies (%) | No. of LR-PCR$^+$ colonies(%) | No. of Southern$^+$ colonies(%) |
|---|---|---|---|---|---|
| SLA1-10 (M) | pPL654 | 127 | 4 (3%) | 0 | 0 |
| PCFF4-2 (M) | pPL657 | 179 | 22 (12%) | 11 (6%) | 2 (1%) |
| PCFF4-3 (M) | pPL657 | 200 | 5 (2.5%) | 1 (0.5%) | 1 (0.5%) |
| PCFF4-6 (F) | pPL657 | 599 | 69 (11.5%) | 18 (3%) | 14 (2%) |

Results for SLA1-10, PCFF4-2, and PCFF4-6 cells are from two individual transfections for each cell; result for PCFF4-3 cells is from one transfection.

of these cells with the pPL657 GT knockout vector. When compared with PCFF4-2 cells, PCFF4-6 cells had a very similar knockout efficiency even though they were transfected with vector made from non-isogenic DNA. As both the PCFF4-2 and PCFF4-6 cell lines were derived from sibling fetuses of the same pregnancy, it is possible that they share a common allele.

We used seven Southern blot–confirmed α1,3GT-knockout single colonies for nuclear transfer. All cells used for nuclear transfer were from the aliquots that had been frozen immediately after the initial 3′ PCR screening. The karyotype of each colony was checked; all had chromosome numbers in a range similar to that of freshly isolated porcine fetal fibroblast cells (~70% of spreads with 38 chromosomes). On average, approximately 150 reconstructed nuclear-transfer embryos were transferred to the oviducts of each estrus-synchronized recipient female. All seven colonies used for nuclear transfer resulted in very high initial pregnancy rates at day 25 (50%–86%) (Table 2). However, all pregnancies established from colonies 657A-A8 and 657A-F12 were lost between days 25 and 45. In contrast, the other five colonies resulted in pregnancy rates in excess of 50% at day 45.

One spontaneously aborted day 38 fetus from a 657A-A8 nuclear transfer recipient was recovered. LR-PCR and Southern blot analysis confirmed that the fetus contained a disrupted α1,3GT locus (data not shown). Southern blot results from 657A-I11 cells showed that the 9-kb knockout band was less intense than the 7-kb endogenous α1,3GT band, indicating that this was most likely a mixed colony containing both wild-type and heterozygous knockout cells. There was some concern that fetuses derived from wild-type cells in the mixed colony could affect the development of, or outcompete, *in utero* fetuses derived from the α1,3GT-knockout cells. To test this, we terminated by hysterectomy a day 32 pregnancy derived from nuclear transfer with 657A-I11 cells. Seven fetuses were recovered, of which six were of normal size and one substantially smaller. Southern blot analysis showed that six of the seven fetuses contained a disrupted α1,3GT locus (Fig. 2A). Notably, it was the smaller fetus (fetus no. 2) that was wild type, and all six normal-sized fetuses contained an α1,3GT knockout allele. These results suggested that there was no discrimination against the heterozygous α1,3GT knockout fetuses *in utero*.

Six live piglets derived from the 657A-I11 cells were born on December 25, 2001. Five were of normal size and weight (Fig. 3); one (no. 2) was stunted, weighing less than 1 pound. Southern blot analysis indicated that five of the six offspring were α1,3GT heterozygous knockouts (Fig. 2B). Again, the one negative (wild-type) piglet was the underdeveloped runt. These results, when considered along with the analysis of the seven day 32 fetuses from 657A-I11 cells, suggested that the colony 657A-I11 was indeed a mixed population contaminated with wild-type cells. All fetuses and offspring obtained from the α1,3GT knockout cells in the 657A-I11 population were developmentally normal. Physical examination of the five knockout piglets at one month of age found no abnormalities. This contrasts with the report by Lai *et al.*[14], in which only four of seven piglets survived for more than a month and three of the surviving piglets had mild physical abnormalities. These differing outcomes may have been due to many factors, including different pig breeds, different condition of cells used for nuclear transfer, and different embryo manipulation methods.

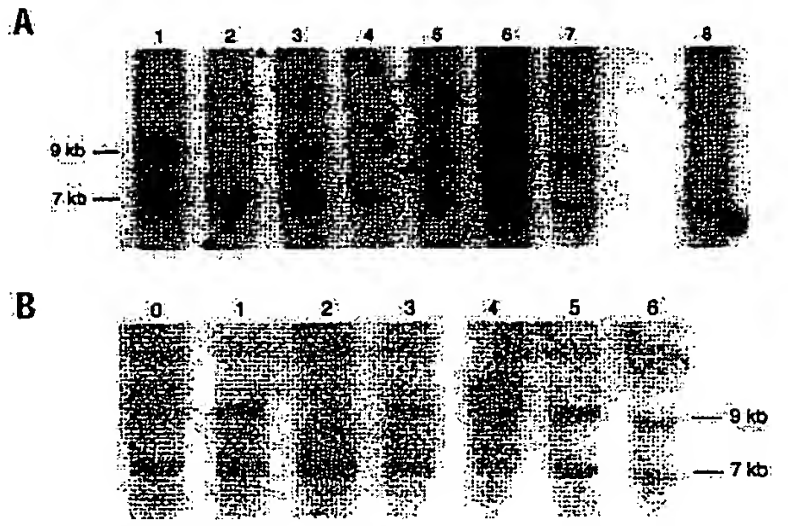


**Figure 2.** Southern blot analysis of α1,3GT gene knockout fetuses and piglets. (A) Southern analysis of DNA from seven day 32 fetuses. Lane 1–7 are *Bst*EII-digested genomic DNA from seven day 32 fetuses derived from 657A-I11 cells. Lane 8 contains normal pig DNA digested with *Bst*EII as a negative control. The 7-kb band represents the endogenous α1,3GT gene and the 9-kb band the disrupted α1,3GT locus. (B) Southern analysis of DNA from six piglets. Lane 0 is the normal pig DNA digested with *Bst*EII. Lanes 1–6 are *Bst*EII-digested DNA from six piglets cloned from 657A-I11 cells. The 7-kb band represents the endogenous α1,3GT gene and the 9-kb band represents the disrupted α1,3GT locus.

We have produced apparently healthy heterozygous α1,3GT knockout piglets by nuclear transfer. We have an additional 16 ongoing second- and third-trimester pregnancies beyond day 45 from two female and three male α1,3GT knockout colonies (Table 2). We expect that most will go to term as we have never lost any pregnancies of cloned pigs after 45 days of gestation (data not shown). The next step is to obtain homozygous pigs with both α1,3GT alleles inactivated. This could be done either through natural breeding of male and female heterozygous knockout animals or through gene targeting with heterozygous knockout cells to disrupt the second α1,3GT allele before a second round of nuclear transfer. As we already have six lines of early-passage heterozygous α1,3GT gene–disrupted fetal fibroblasts, obtained from the day 32 657A-I11 fetuses, it will probably be considerably faster to create the second knockout in these cells *in vitro* and obtain homozygous knockout animals by nuclear transfer. Natural breeding to homozygosity will also be used, but this method will take considerably longer because of the gestation time of the male knockout clones *in utero* and the time to sexual maturity.

Live births have been reported in cattle from recloning experiments that used fibroblasts obtained from cloned fetuses[17]. Recloning experiments by our group, using wild-type porcine fetal fibroblasts derived from a day 40 cloned fetus, have shown an 80% pregnancy rate at day 45 (PPL Therapeutics, unpublished data). These data suggest that it will be feasible to obtain homozygous α1,3GT knockout pigs by a second knockout and recloning

**Table 2. Summary of nuclear transfer results from α1,3GT knockout primary fibroblast cells**

| Nuclear donor cell line | PCFF4-6 | PCFF4-6 | PCFF4-6 | PCFF4-6 | PCFF4-2 | PCFF4-2 | PCFF4-3 |
|---|---|---|---|---|---|---|---|
| Cell clone | 657A-I11 | 657A-A8 | 657A-F12 | 657A-I6 | 657F-J10 | 657F-C11 | 657B-K8 |
| Sex | F | F | F | F | M | M | M |
| Embryos transferred to recipients | 1097 | 825 | 591 | 976 | 1009 | 775 | 1105 |
| Recipients | 7 | 5 | 4 | 6 | 6 | 4 | 7 |
| Pregnancies at day 25 | 6 | 3 | 2 | 5 | 5 | 2 | 6 |
| Pregnancies at day 45 | 3[a] | 0 | 0 | 4 | 4 | 2 | 4[b] |
| Piglets at birth[c] | 6 | 0 | 0 | Pending | Pending | Pending | Pending |

[a]Based on 6 recipients since one day 32 pregnancy was terminated for fetal fibroblast isolation. [b]Based on 6 recipients since one day 39 pregnancy was terminated for fetal fibroblast isolation. [c]As dated on January 25, 2002. Six piglets were born from one 657A-I11 recipient on December 25, 2001. Another 16 recipients beyond day 45 of pregnancy are due after January 25, 2002.

Figure 3. Five α1,3GT gene knockout piglets at 2 weeks of age.

strategy. In mice, the homozygous knockout of α1,3GT gene is not an embryonic lethal mutation, although such mice have developed cataracts[6]. Pig cells express significantly more α1,3Gal epitopes on their surface than do mouse cells, and it has therefore been proposed that α1,3GT may have some additional, unknown role in pigs[18]. Although heterozygous α1,3GT knockout pigs are developmentally normal, it is not known if complete deletion of both alleles of α1,3GT will be more (or less) problematic in pigs than in mice.

Complete removal of the α1,3Gal epitope, the major xenoantigen, combined with transgenic expression of complement regulatory proteins should prevent the hyperacute rejection of pig xenografts even in the presence of a low background of non-α1,3Gal xenoantigens. The success of xenotransplantation will also depend on risk assessment of safety factors such as porcine endogenous retroviruses and on the development of strategies that address delayed vascular and T-cell-mediated rejection. Together, these approaches may provide a near-term solution to the chronic shortage of human organs (such as heart and kidneys) and valuable tissues such as insulin-producing islet cells.

## Experimental protocol

Isolation and transfection of primary porcine fetal fibroblasts. PCFF4-1 to PCFF4-10 fetal fibroblast cells were isolated from 10 fetuses of the same pregnancy at day 33 of gestation. After removing the head and viscera, fetuses were washed with Hanks' balanced salt solution (HBSS; Gibco-BRL, Rockville, MD), placed in 20 ml of HBSS, and diced with small surgical scissors. The tissue was pelleted and resuspended in 50-ml tubes with 40 ml of DMEM and 100 U/ml collagenase (Gibco-BRL) per fetus. Tubes were incubated for 40 min in a shaking water bath at 37°C. The digested tissue was allowed to settle for 3–4 min and the cell-rich supernatant was transferred to a new 50-ml tube and pelleted. The cells were then resuspended in 40 ml of DMEM containing 10% fetal calf serum (FCS), 1× nonessential amino acids, 1 mM sodium pyruvate (Gibco-BRL), and 2 ng/ml basic fibroblast growth factor (bFGF; Roche Molecular Biochemicals, Indianapolis, IN), and seeded into 10-cm dishes. All cells were cryopreserved upon reaching confluence. SLA1-1 to SLA1-10 cells were isolated from 10 fetuses at day 28 of pregnancy. Fetuses were mashed through a 60-mesh metal screen (Sigma, St. Louis, MO) using curved surgical forceps slowly so as not to generate excessive heat. The cell suspension was then pelleted and resuspended in 30 ml of DMEM containing 10% FCS, 1× nonessential amino acids, 2 ng/ml bFGF, and 10 μg/ml gentamycin. Cells were seeded in 10-cm dishes, cultured one to three days, and cryopreserved. For transfections, 10 μg of linearized vector DNA was introduced into 2 million cells by electroporation. Forty-eight hours after transfection, the transfected cells were seeded into 48-well plates at a density of 2,000 cells per well and were selected with 250 μg/ml of G418 (Gibco-BRL).

Knockout vector construction. Two α1,3GT knockout vectors, pPL654 and pPL657, were constructed from isogenic DNA of two primary porcine fetal fibroblasts, SLA1-10 and PCFF4-2 cells. A 6.8-kb α1,3GT genomic fragment, which includes most of intron 8 and exon 9, was generated by PCR from purified DNA of SLA1-10 cells and PCFF4-2 cells, respectively. The unique *Eco*RV site at the 5′ end of exon 9 was converted into a *Sal*I site and a 1.8-kb *IRES-neo*-poly A fragment was inserted into the *Sal*I site. IRES (internal ribosome entry site) functions as a translation initial site for neo protein. Thus, both vectors have a 4.9-kb 5′ recombination arm and a 1.9-kb 3′ recombination arm (Fig. 1A).

3′ PCR and long-range PCR. Approximately 1,000 cells were resuspended in 5 μl embryo lysis buffer (ELB) (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P-40, 0.4 mg/ml proteinase K), incubated at 65°C for 15 min to lyse the cells, and heated to 95°C for 10 min to inactivate the proteinase K. For 3′ PCR analysis, fragments were amplified using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) in 25 μl reaction volume with the following parameters: 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. For LR-PCR, fragments were amplified by using TAKARA LA system (Panvera/Takara, Madison, WI) in 50 μl reaction volume with the following parameters: 30 cycles of 10 s at 94°C, 30 s at 65°C, 10 min + 20 s increase/cycle at 68°C; and one final cycle of 7 min at 68°C. 3′ PCR and LR-PCR conditions for purified DNA was same as for cells except that 1 μl of purified DNA (30 μg/ml) was mixed with 4 μl ELB.

Southern blot analysis of cell samples. Approximately $10^6$ cells were lysed overnight at 60°C in lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% (w/v) Sarcosyl, 1 mg/ml proteinase K) and the DNA precipitated with ethanol. The DNA was then digested with *Bst*EII and separated on a 1% agarose gel. After electrophoresis, the DNA was transferred to a nylon membrane and probed with the 3′-end digoxigenin-labeled probe. Bands were detected using a chemiluminescent substrate system (Roche Molecular Biochemicals).

Southern blot analysis of pig tissues. Fetal tissues and piglet tails were lysed overnight at 60°C in a shaking incubator with approximately 1 ml lysis solution (50 mM Tris, pH 8.0, 0.15 M NaCl, 10 mM EDTA, 1% SDS, 25% sodium perchlorate, 1% 2-mercaptoethanol, and 200 μg/ml proteinase K) per 175 mg tissue. DNA was subjected to phenol/chloroform extraction and precipitated with isopropyl alcohol. Resolubilized DNA was treated with RNase A (1 mg/ml) and RNase T1 (1,000 U/μl) at 37°C for 1 h, with proteinase K (20 mg/ml) at 55°C for 1 h, then extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer. About 10 mg DNA was digested with *Bst*EII and separated on an 1% agarose gel. Following electrophoresis, the DNA was transferred to a nylon membrane and probed with the 3′-end digoxigenin-labeled probe. Bands were detected using a chemiluminescent substrate system.

Nuclear transfer procedure. Enucleation of *in vitro*–matured oocytes (BioMed, Madison, WI) was begun between 40 and 42 h post-maturation as described previously[11]. A single fibroblast cell was placed under the zona pellucida in contact with each enucleated oocyte. Fusion and activation were induced by application of an AC pulse of 5 V for 5 s followed by two DC pulses of 1.5 kV/cm for 60 μs, each using an ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA). Fused embryos were cultured in NCSU-23 medium for 1–4 h at 38.6°C in a humidified atmosphere of 5% $CO_2$, and then transferred to the oviduct of an estrus-synchronized recipient gilt. Crossbred gilts (large white/Duroc/landrace) (280–400 lbs) were synchronized as recipients by oral administration of 18–20 mg Regu-Mate (Altrenogest, Hoechst, Warren, NJ) mixed into their feed. Regu-Mate was fed for 14 consecutive days. Human chorionic gonadotropin (hCG, 1,000 units; Intervet America, Millsboro, DE) was administered intramuscularly 105 h after the last Regu-Mate treatment. Embryo transfers were done 22–26 h after the hCG injection.

*Acknowledgments*

*We thank B. Gragg, W. Lucero, T. Akers, H. Bishop, and J. McPherson for technical contributions to embryo transfer and animal husbandry; A. Garst and J. Hencke for help in nuclear transfer; C. Kolke and T. E. Starzl at the University of Pittsburgh for providing us with porcine α1,3GT gene genomic sequence; M. Moore, A. Kind, and A. Schnieke for valuable contributions in discussions; and staff at the Virginia Maryland Regional Veterinary College for prenatal care of some of the nuclear transfer recipients and physical examination of piglets. This research was funded in part by a grant from the National Institute of Standards and Technology (NIST) Advanced Technology Program (ATP).*

*Competing interests statement*

*The authors declare that they have no competing financial interests.*

Received 8 January 2002; accepted 22 January 2002

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Transplant Immunology 8 (2000) 129–137

Transplant Immunology

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# Anti-galactose-α(1,3) galactose antibody production in α1,3-galactosyltransferase gene knockout mice after xeno and allo transplantation

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Received 25 February 2000; accepted 30 May 2000

**Abstract**

Antibodies (Abs) that mediate the hyperacute rejection and acute vascular rejection delayed xenograft rejection of pig organs in humans and Old World primates are predominantly directed at a single carbohydrate epitope, galactose-α1,3-galactose (α1,3Gal). The T-cell dependence of elicited anti-α1,3Gal Ab responses in humans and Old World primates is controversial. In this study we have characterized anti-α1,3Gal Ab production in mice with disrupted α1,3-galactosyltransferase genes (GT-Ko mice) and determined the T-cell dependence of anti-α1,3Gal Ab responses, following xenograft and allograft transplantation. GT-Ko mice produce natural anti-α1,3Gal IgM and IgG in an age-dependent manner, however, these Abs could not elicit hyperacute rejection nor affect the rate of cardiac xenograft (3–5 days) or allograft rejection (7–9 days). Transplantation of xenogeneic Lewis rats hearts elicited modest anti-α1,3Gal Ab, but vigorous xenoAb responses. The anti-α1,3Gal Ab response was restricted to the IgM and IgG3 subclass while the xenoAb response comprised IgM and all four IgG subclasses. Transplantation of allogeneic C3H hearts elicited weak anti-α1,3Gal Ab responses that were primarily IgM, but vigorous alloAb responses. Despite the restriction of elicited anti-α1,3Gal Ab responses to the IgM and IgG3 isotypes, these responses are T-cell dependent. The ability of allografts to elicit weak anti-α1,3Gal but strong allo-Ab responses, can be explained by the dependence of α1,3Gal-specific B cells on cognate help from T cells. © 2000 Elsevier Science S.A. All rights reserved.

*Keywords:* Xenotransplantation; Galactosyltransferase knock-out; Mice; Anti-α1,3gal antibodies; Xenoantibodies; Humoral responses

## 1. Introduction

The inadequate supply of human organs has created a strong interest in the use of non-primate organs for clinical transplantation but vigorous immune reactions prevent their current use in humans. The expression of transgenic human complement regulatory genes confers to porcine organs resistance to complement-mediated hyperacute rejection 1–4. However, significant immune responses directed against the xenograft remain, and the xenografts are eventually rejected by a process collectively referred to as acute vascular rejection (AVR) or delayed xenograft rejection (DXR). The production of XAbs is a dominant feature of AVR DXR and there is increasing evidence that elicited XAbs are the primary mediators of AVR DXR 5,6.

The hyperacute rejection of porcine organs by hu-

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0966-3274 00 $ - see front matter 
PII: S0966-3274(00)00017-4

mans or Old World monkeys is elicited by naturally occurring anti-pig Abs that are directed to a single carbohydrate epitope, α1,3Gal 7,8. The elicited immune response following exposure to porcine tissues also appears to be directed primarily at the α1,3Gal epitope 9–12. There are conflicting data as to whether natural and elicited anti-α1,3Gal Ab production following xenotransplantation is T cell-dependent. On one hand, the resistance to conventional T cell immunosuppression and the lack of elicited Abs that recognize new antigenic determinants are consistent with a T-independent anti-carbohydrate response, while observations of vigorous anti-α1,3Gal IgG production and increased affinities for α1,3Gal suggest that it may be T-cell dependent 10–13.

Opportunities to study anti-α1,3Gal responses have historically been restricted to Old World monkeys or humans 14. However, the recent generation of mice with disrupted α1,3-galactosyltransferase (GT) genes, GT-Ko mice, provides an opportunity to study the immunological basis of the anti-α1,3Gal Ab response 15–21. In this study we have characterized anti-α1,3Gal Ab in GT-Ko mice and determined the T-cell dependence anti-α1,3Gal Ab response following xenograft and allograft transplantation.

## 2. Materials and methods

### 2.1. Animals

GT-Ko mice were produced by homologous recombination of the α1,3-galactosyltransferase gene in 129 ES cells, recombinant clones were transferred into (C57BL 6 X DBA 2)F1 mice, and a homozygous line was established on a background of C57BL 6, DBA 2 and 129SvSn strains 16. These mice were obtained from Dr John Lowe (Howard Hughes Medical Institute, U. Michigan, Ann Arbor, MI, USA) and maintained at the Rush Presbyterian St. Luke's Medical Center. Ten to 16-day-old Lewis rats (Harlan, Walkersville, MD, USA) or 8–10-week-old C3H mice (Taconic, Germantown, NY, USA) were used as heart donors.

### 2.2. Transplantation

Heterotopic mouse or rat hearts were transplanted into the abdomen of the recipient by anastomosing the donor aorta and recipient aorta, and the donor pulmonary artery and recipient inferior vena cava as previously described 22. Human serum and or baby rabbit complement (Pel-Freeze, Rogers, AR, USA) was introduced by intravenous injection into the penile vein or tail vein, and grafts were observed for 30–60 min before closing. The heart grafts were palpated hourly for the first day, then daily until rejection. Rejection is defined as complete cessation of pulsation.

### 2.3. Serum

Human serum was obtained from one individual with high titers of anti-α1,3Gal Abs. Pooled GT-Ko mouse serum was harvested after xenograft or allograft rejection, and heat-inactivate at 56°C for 30 min. Anti-α1,3Gal Abs were depleted from pooled GT-Ko mouse serum by affinity chromatography using Galα(1,3) Galβ(1,3)GluNAc conjugated resin (Nextran, Princeton, NJ, USA).

### 2.4. Quantification of anti-α1,3Gal Ab titers

Anti-α1,3Gal Ab titers were determined by enzyme-linked immunosorbent assay (ELISA) using BSA-α1,3Gal (V-Labs, Covington, LA, USA) as a specific antigen (10 μg ml), and BSA (Sigma, St. Louis, MO, USA) as non-specific control. For competition experiments, serial dilutions of mouse serum were preincubated with 10 mM α1,3Gal trisaccharide (Cytel, San Diego, CA, USA) for 1 h at 4°C. BSA-α1,3Gal- or BSA-coated plates (Costar, Corning, NY, USA) were pre-blocked with 5% BSA PBS, then serum was added. After 1 h, plates were washed, blocked with 5% BSA PBS then incubated with horse radish peroxidase (HRP)-conjugated anti-mouse IgM or anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA). For quantifying the anti-α1,3Gal IgG subclass, biotinylated isotype-specific monoclonal Abs (PharMingen, San Diego, CA, USA) and Strepavidin-HRP (Dako Corp., Carpenteria, CA, USA) were used. Standards of mouse IgM, IgG1, IgG2a, IgG2b and IgG3 were purchased from PharMingen, serially diluted and added to each plate at the same time the plates were coated with BSA-α1,3Gal or BSA. The wells coated with standards were subject to the same treatment as experimental wells, except that no sera were added. The relative concentrations of anti-α1,3Gal IgG subclasses in the sera were determined by comparison of o.d. to the standard curve of each IgG subclass.

### 2.5. Quantification of xeno-specific and allo-specific Ab titers

Xeno-specific and allo-specific Ab titers were determined by flow cytometry as previously described 22. Briefly, 1 50 or 1 100 dilutions of mouse serum were pre-incubated with α1,3Gal trisaccharide (Cytel, San Diego, CA, USA) for 1 h at room temperature. Then $10^6$ Lewis rat erythrocytes or C3H lymphocytes were incubated with the mouse serum for 1 h at room temperature. The cells were washed and incubated with phycoerythrin-conjugated anti-mouse IgM (Jackson Im-

munoresearch) or fluorescein-conjugated anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA). For identifying the IgG subclasses, biotinylated isotype-specific monoclonal Abs and Strepavidin-FITC (PharMingen, San Diego, CA, USA) were used. The mean fluorescence intensity (MFI) was determined by flow cytometry (Ortho Cytoron Absolut, Ortho Diagnostic Systems, Raritan, NJ, USA). The percent of xeno or allo Ab response that is anti-α1,3Gal-specific was calculated from the MFI for unblocked serum and α1,3Gal trisaccharide-blocked serum. These values were converted to linear scale and calculated using the equation:

%α1,3Gal reactive Abs

$$(MFI_{unblocked} \quad MFI_{blocked}) \times 100 \quad MFI_{unblocked}.$$

### 2.6. Histology and immunohistochemistry

Heart grafts were surgically removed and snap-frozen in Tissue-Tek OCT (Sakura Finetek USA, Torrence, CA, USA) using liquid nitrogen. The hearts were sectioned (5 μm) and stained with hematoxylin and eosin (H&E). Other sections for immunohistochemical staining were subject to the standard avidin–biotin peroxidase (ABC) method as previously described 22. Primary Abs of anti-mouse IgM (R4-22), anti-mouse IgG (R3-34), anti-TCRαβ (H57-597), anti-CD4 (H129-19), anti-CD8α (53-6.7), Mac-1(M1 70), and anti-CD45RB (RA3-6B2) were purchased from PharMingen, biotinylated goat anti-mouse IgG and HRP-conjugated-streptavidin were from Jackson Immunochem. Labs. (West Grove, PA, USA) and Zymed Labs (South San Francisco, CA, USA), respectively. Immunostaining was developed with chromogen, 3,3′-diaminobenxidine solution, and counterstained with Mayer's hematoxylin.

## 3. Results

### 3.1. Relationship between age of GT-Ko mice and titers of anti-α1,3Gal Abs

In humans, maternally-derived anti-α1,3Gal Abs decrease to the lowest levels at 3–6 months of age, then natural α-α1,3Gal Ab titers gradually rise to steady state levels in 2–4 years 23. We first tested whether a similar increase in anti-α1,3Gal Abs occurs in GT-Ko mice maintained in our facility. We quantified the titers of anti-α1,3Gal Abs with an ELISA using immobilized α1,3Gal conjugated to BSA as a specific substrate, and BSA as a non-specific control. The specificity of the assay was further confirmed by the ability of 10 mM α1,3Gal-trisaccharide to abrogate IgM and IgG binding to BSA-α1,3Gal. We observed an age-dependent increase in the titers of anti-α1,3Gal Abs — there was approximately a twofold increase in anti-α1,3Gal IgM titers between the first (6–13 weeks) and second (13–22 weeks) age group, and a greater than fourfold between the first and third ( 22 weeks) age group (Fig. 1A). A similar increase was observed with anti-α1,3Gal IgG titers (Fig. 1B). The titers of anti-α1,3Gal IgM increased before the titers of anti-α1,3Gal IgG; anti-α1,3Gal IgM could be detected in the serum in 6–13 weeks old GT-Ko mice whereas anti-α1,3Gal IgG was first detected in GT-Ko mice at 13–22 weeks of age.

### 3.2. Effect of pre-formed anti-α1,3Gal Abs on xenograft rejection

It has been established that pre-formed anti-α1,3Gal Abs in baboons or humans elicit hyperacute rejection of porcine grafts. We therefore asked whether Lewis rat hearts could be hyperacutely rejected by the pre-formed anti-α1,3Gal Abs in GT-Ko mice. Cohorts of GT-Ko mice were divided into three age groups as defined in Fig. 1. Heterotopically transplanted Lewis

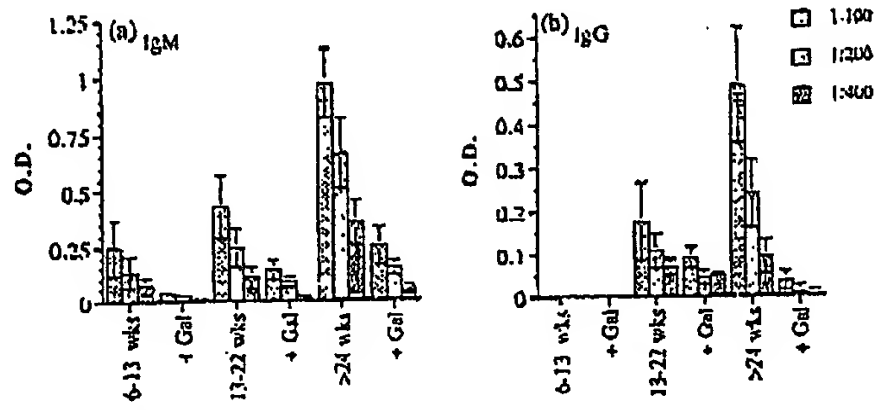


Fig. 1. Titers of anti-α1,3Gal Abs increase with age in GT-Ko mice. ELISA plates were coated with BSA-α1,3Gal or BSA (10 μg ml). Serum was obtained from GT-Ko mice at the indicated weeks after birth, and serially diluted (4×) from 1:100. Bound anti-α1,3Gal IgM or IgG were detected with HRP-conjugated anti-mouse Abs. Gal: Serum was preincubated with 2 mM α1,3Gal-trisaccharide for 1 h at room temperature before addition to ELISA plates. Data are presented as o.d. from BSA-α1,3Gal-coated plates subtracted from O.D. from BSA-coated plates ($N$ 5–9 per group) group and bars represent S.E.M.

Table 1
Effect of preformed antibodies on xenograft and allograft rejection

| Recipient | Donor | Graft survival (days) | Mean (days) | S.E.M. |
|---|---|---|---|---|
| 6–12 weeks old GT-Ko mice | Lewis rat | 4, 4, 5, 5, 5, 5, 5, 5, 5 | 4.8 | 0.4 |
| 13–22 weeks old GT-Ko mice | Lewis rat | 3, 4, 4, 4, 4, 5, 5 | 4.1 | 0.6 |
| 22 weeks old GT-Ko mice | Lewis rat | 4, 4, 4, 4, 4, 5, 5, 5, 5 | 4.4 | 0.5 |
| 8–12 weeks old WT-F1 mice | Lewis rat | 6, 6, 6 | 6.0 | 0 |
| 15 weeks old GT-Ko mice | C3H | 7, 7, 7, 7, 8, 8, 8, 9 | 7.6 | 0.7 |
| 8–12 weeks old WT-F1 mice | C3H | 8, 8, 8, 8, 8 | 8.0 | 0 |

rat hearts were rejected in 3–5 days, with no observable effect of age on the rate of rejection (Table 1). Lewis rat hearts were rejected by control (C57BL 6 DBA 2)F1 mice in 6 days. The pathological features were consistent with humoral rejection, namely hemorrhage, edema, thrombosis, myocyte necrosis, IgM deposition and a modest cellular infiltrate that was predominantly macrophages (Fig. 2 and data not shown).

The inability of pre-formed anti-α1,3Gal Abs to elicit hyperacute rejection could be due to insufficient preformed anti-α1,3Gal Abs or to the ability of the rat complement regulatory proteins to inhibit mouse complement activation. To test these possibilities, we assessed the ability of heterologous complement, from rabbit or human serum, to induce hyperacute rejection of Lewis rat hearts when transplanted into GT-Ko mice ( 12 weeks old). In three of four individuals 0.2 ml of rabbit serum, which does not contain anti-α1,3Gal Abs, induced hyperacute or accelerated rejection of Lewis rat hearts in six of seven hearts (Table 2). Higher doses of rabbit complement were toxic (data not shown). Pooled human serum (0.25 ml), which contains both anti-α1,3Gal Abs and heterologous complement, induced hyperacute rejection of all four Lewis rat hearts (Table 2). Heat inactivation of complement completely abrogated the ability of human serum to induce hyperacute rejection, while, co-administration of heat inactivated human serum with rabbit complement induced hyperacute rejection of Lewis rat hearts (Table 2). These data suggest that the GT-Ko mice can elicit hyperacute or accelerated rejection of Lewis rat hearts if heterologous complement regulatory and or additional anti-α1,3Gal Abs were present. These results corroborate and extend the observations made by McKenzie et al. 18.

### 3.3. Anti-α1,3Gal Ab production following xenotransplantation

The elicited xenoAb response following transplantation of porcine grafts into Old World primates and humans are directed primarily at anti-α1,3Gal epitopes. We tested the dominance of anti-α1,3Gal Abs in the Lewis rat-to-GT-Ko mouse model. Xenograft rejection was accompanied by an increase in anti-α1,3Gal Abs (Fig. 3). The titers of anti-α1,3Gal IgM and IgG increased four to eightfold and eight to 16-fold, respectively, at the time of rejection (Fig. 3). The isotype of circulating anti-α1,3Gal Abs was determined by ELISA and compared to a standard curve of purified mouse IgM or IgG1, IgG2a, IgG2b or IgG3. The elicited anti-α1,3Gal Abs were predominantly IgM (83.0

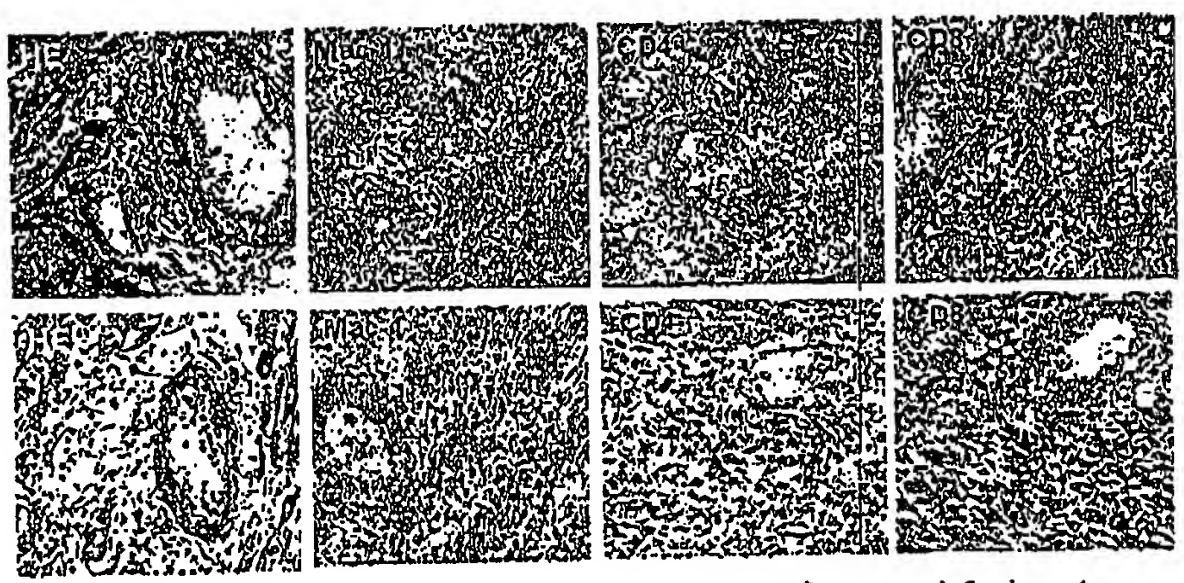


Fig. 2. Histology and immunohistochemistry of rejected xenografts (top row) or allografts (bottom row). Sections of xenografts, harvested at the time of rejection, were stained with H&E, Mac-1, CD4 and CD8α.

Table 2
Hyperacute rejection of Lewis rat hearts transplanted into GT-Ko mice

| Treatment | | Vol. (ml) | Graft survival | Mean | S.E.M. |
|---|---|---|---|---|---|
| None | | | 4, 4, 5, 5, 5, 5, 5, 5 days | 4.8 | 0.4 days |
| Rabbit serum | | 0.2 | 4, 4, 4.5, 4.5, 18, 18, 120 h | 24.7 | 16.1 h |
| Human serum | | 0.25 | 8, 15, 18, 18 min | 14.8 | 2.4 min |
| Heat-inactivated human serum | | 0.5 | 1, 3, 4, 4, 4, 5 days | 3.5 | 0.7 days |
| Heat-inactivated human serum | Rabbit serum | 0.25 0.2 | 1, 2, 2, 2 h | 1.75 | 0.3 h |

μg ml) and IgG3 (108.7 μg ml) (Fig. 3A). At 20 days post-transplantation with Lewis rat hearts, less than 5 μg ml of anti-α1,3Gal IgG2a and 2b and 1μg ml anti-α1,3Gal IgG1 were detected in the serum of GT-Ko mice (Fig. 4A).

We next defined what portion of the XAb response in GT-Ko mice was α1,3Gal specific. The preformed natural XAbs in GT-KO mice are largely α1,3Gal specific since binding of these XAbs to Lewis RBC targets were inhibited by 10 mM α1,3Gal trisaccharide (Fig. 5). Following transplantation of Lewis rats, the titers of xenoreactive IgM increased 4.6–54-fold, depending on the pre-transplant levels of xenoreactive IgM, while the titers of xenoreactive IgG increased 6.2–11-fold (Fig. 5). The subclasses of circulating xenoreactive IgG were IgG2a IgG3 IgG1 IgG2b in GT-Ko mice at 20 days after transplantation with Lewis rat hearts (Fig. 4A). Xenoreactive IgM and IgG binding to Lewis RBC targets was marginally reduced ( 30%) by 10 mM α1,3Gal-trisaccharide suggesting that the anti-α1,3Gal Ab response was a minor component of the total xenoantibody response in GT-Ko mice (Fig. 5). To further test this conclusion, we pooled serum from GT-Ko mice with rejected Lewis rat hearts, and depleted the serum of anti-α1,3Gal Abs. Intravenous injection of 0.2 ml of pooled heat-inactivated serum, either non-depleted or anti-α1,3Gal Ab-depleted, were able to induce hyperacute rejection of freshly transplanted Lewis rat hearts (Table 3). These observations confirm that anti-α1,3Gal Abs play a minor role in xenograft rejection in this Lewis rat-to-GT-Ko mouse model.

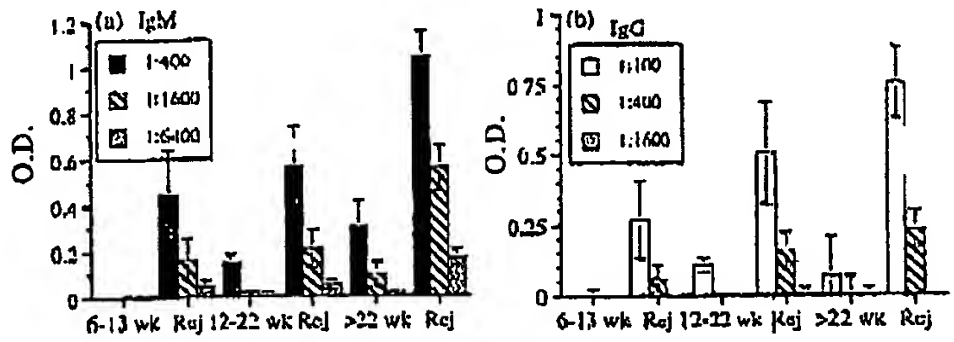


Fig. 3. Titers of anti-α1,3Gal IgM and IgG in GT-Ko mice increase at the time of xenograft rejection. Serum was diluted as indicated, the ages of GT-Ko mice at time of transplant and at rejection (Rej) are indicated on the *X*-axis. Anti-α1,3Gal Ab titers were quantified by ELISA as described for Fig. 1.

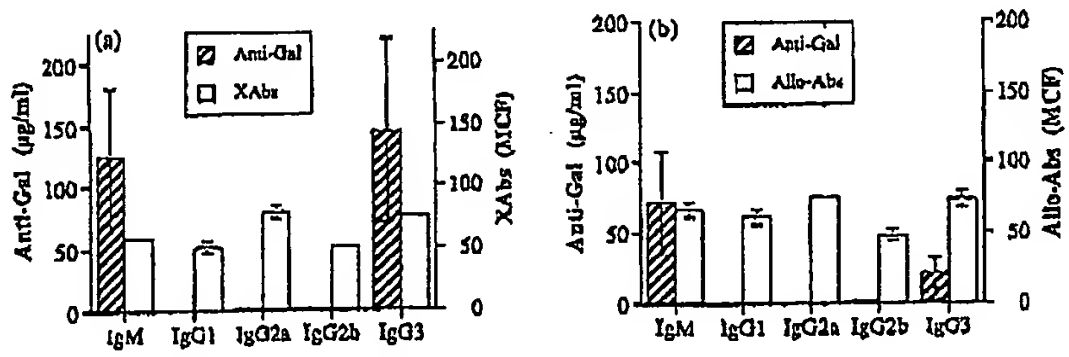


Fig. 4. Relative concentrations of anti-α1,3Gal Abs, xenoAbs and alloAbs at 20 days post-transplantation. Anti-α1,3Gal Abs were quantified by ELISA as described in Section 2. XAbs and alloAbs were quantified by flow cytometry using Lewis erythrocytes and C3H lymphocytes, respectively. Data are presented as mean o.d. (ELISA) or mean channel fluorescence (xenoAb and alloAb) of 8–9 individuals S.E.M.

Table 3
Role of anti-Gal Abs in hyperacute rejection

| | Vol. (ml) | Graft survival | Mean S.E.M. |
|---|---|---|---|
| *Graft: Lewis rat hearts* | | 4, 4, 4, 4, 4, 5, 5, 5, 5 days | 4.4 0.2 days |
| None | | | |
| HI-Pooled Xabs | 0.2 | 5, 7, 7, 8 min | 6.8 0.6 min |
| HI-Anti-Gal depleted pooled Xabs | 0.25 | 15, 15, 20, 20 min | 17.5 1.4 min |
| Human serum | 0.25 | 8, 15, 18, 18 min | 14.8 2.4 min |
| *Graft: C3H hearts* | | 7, 7, 7, 7, 8, 8, 8, 9 days | 7.6 0.7 days |
| None | | | |
| Pooled Allo-Abs | 0.2 | 8 days | 8 days |
| Pooled Allo-Abs | 0.4 | 8, 8 days | 8 days |
| Human serum | 0.4 | 9, 12, 15, 20, 20 min | 15.2 2.2 min |

### 3.4. Comparison of anti-α1,3Gal Ab production following xenograft or allograft transplantation

Anti-α1,3Gal Ab production in humans are only elicited following xenotransplantation, however, a number of early studies with GT-Ko mice have used syngeneic or allogeneic wild-type grafts to stimulate anti-α1,3Gal production 17,18,24. We hypothesized that allografts may be less effective at stimulating anti-α1,3Gal Ab production in GT-Ko mice compared to xenografts. To test this hypothesis, we transplanted allogeneic C3H hearts into GT-Ko mice ( 12 weeks old). Allogeneic C3H hearts were rejected by GT-Ko mice in 7.6 days, respectively (Table 1). In contrast to xenografts, histological examination revealed that allografts were rejected by classical cellular responses (Fig. 2). There was significant CD8α and CD4 T cell and macrophage infiltration, extensive IgM but minimal IgG deposition in the allografts (Fig. 2 and data not shown). The secondary role of alloAbs in allograft rejection was consistent with the inability of immune serum, pooled from GT-Ko mice with rejected allografts, to induce the hyperacute rejection of freshly transplanted allografts (Table 3).

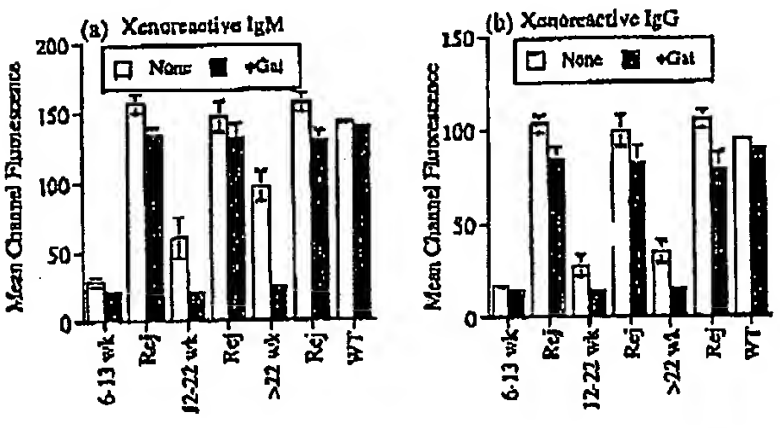


Fig. 5. Titers of xenoreactive IgM and IgG in serum of GT-Ko mice with rejected Lewis rat hearts. Serum was obtained from GT-Ko mice at the indicated ages, before transplantation or at the time of rejected Lewis rat heart. Serum was diluted 1 100 for the assay, and Ab binding to Lewis rat erythrocytes was partially blocked by 10 mM α1,3Gal trisaccharide ( Gal). The α1,3Gal-independent XAb response in wild-type (C57BL 6JXDBA 2J) F1 mice (WT) was not inhibited by the α1,3Gal trisaccharide. Data are presented as mean channel fluorescence of 10–12 mice group and bars represent S.E.M.

We next quantified the amount of alloreactive and anti-α1,3Gal IgM and IgG subclasses following allotransplantation. The IgG subclasses for alloAbs were IgG2a IgG3 IgG1 IgG2b (Fig. 4B). As with the xenografts, the predominant anti-α1,3Gal Abs were IgM (8.14 μg ml) and IgG3 (15.33 μg ml) (Fig. 4B). However, the levels of elicited anti-α1,3Gal Abs following allotransplantation were 10.2 and 7.1-fold lower, for IgM and IgG3, respectively, than observed for xenotransplantation. The low levels of allograft-elicited anti-α1,3Gal Abs was confirmed by the observations that α1,3Gal-trisaccharides (10 mM) were unable to reduce alloAbs binding to C3H lymphocyte targets (data not shown).

## 4. Discussion

The generation of the GT-Ko mouse provides unique opportunities to investigate the immunology of anti-α1,3Gal responses in vivo. We have conducted a detailed characterization of the anti-α1,3Gal response by GT-Ko mice prior to and following transplantation with wild-type allografts or xenografts. Naive GT-Ko mice produce anti-α1,3Gal Abs in an age-dependent manner; anti-α1,3Gal IgM is detected before anti-α1,3Gal IgG. These preformed anti-α1,3Gal Abs are not able to significantly alter the tempo of graft rejection, however, the introduction of heterologous complement or anti-donor Abs into GT-Ko mice can induce the hyperacute rejection of Lewis rat hearts. Thus, the inability of preformed anti-α1,3Gal Abs in GT-Ko mice to induce hyperacute rejection of transplanted rat hearts is due to the ability of complement regulatory proteins on rat hearts to control the activation of mouse complement, and to the relatively low levels of preformed anti-α1,3Gal Abs.

A recent report by Apostolopoulos et al. suggested

that pre-existing anti-α1,3Gal Abs inhibited the normal cellular immune response to MUC antigens and favored the development of a humoral one 25. We tested whether a similar effect would be seen in our model, and whether allograft rejection would be converted from a T cell-mediated process to Ab-mediated one. We observed that pre-formed anti-α1,3Gal Abs had no significant effect on the nature of allograft rejection, and that all allografts demonstrated characteristic signs of T-cell mediated rejection.

One surprising observation with our allo-transplantation model was that only weak anti-α1,3Gal Abs responses were elicited, even though alloAb production was vigorous. Preformed anti-α1,3Gal Ab production is thought to occur continuously throughout life. It has been suggested that α1,3Gal-antigenic stimulation is constantly provided by bacteria within the normal intestinal flora carrying the α1,3Gal epitope on lipopolysaccharides and other cell wall components 26. Thus, we had expected that transplantation of α1,3Gal-expressing allografts would elicit a rapid and vigorous anti-Gal Ab response. However, we observed that the tempo of the anti-α1,3Gal response following allograft as well as xenograft transplantation was relatively weak, restricted to the IgM and IgG3 subclasses, and comparable to naive Ab responses 27.

The relatively weak anti-α1,3Gal Ab responses following allotransplantation in GT-Ko mice with preformed anti-α1,3Gal Abs can be explained by the observations that anti-α1,3Gal Ab responses are T-cell dependent, and require cognate interactions between peptide-reactive T cells and anti-α1,3Gal-producing B cells 28,29. Following allotransplantation, allo-reactive T cells become activated by antigenic peptides presented by class II molecules on antigen presenting cells (Fig. 6). These allopeptides active alloreactive T cells that provide help to allo-reactive B cells, thus stimulating vigorous T-dependent alloAb responses. These antigenic peptides are mostly likely to be derived from MHC and minor transplantation antigens. In contrast, glycoproteins that express high levels of the α1,3Gal epitope are most likely to bind to anti-α1,3Gal-producing B cells, be taken up, processed and the resulting peptides presented by MHC class II molecules. However, many of the molecules from C3H mice that express high levels of the α1,3Gal epitope, such as laminin and thyroglobulin, would be considered 'self' antigens for GT-Ko mice. The skewed presentation of these 'self-peptides', instead of 'allo-peptides', by α1,3Gal-specific B cells would result in insufficient T-cell help and weak T-dependent anti-α1,3Gal Ab responses.

The situation is different after xenograft transplantation, since most molecules that are not immunogenic for individuals within the same species are immunogenic for individuals of another species. Thus, xenoantigens that express high levels of α1,3Gal epitopes will be preferentially taken up and presented on α1,3Gal-specific B cells or other APC to T cells capable of recognizing these peptides (Fig. 6). These T cells become activated and can provide cognate help to β1,3Gal-specific B cells, stimulating them to undergo class switch and affinity maturation.

Finally, we observed that in this xenotransplantation model, the anti-α1,3Gal Ab response is only a minor portion of the total elicited XAb response. This observation is in contrast to humans where the anti-α1,3Gal response can represent up to 80% of the xenoAb response 10–12, and appear to be the major factor in hyperacute and acute vascular xenograft rejection 6. One explanation for this discrepancy is that in pig-to-baboon xenotransplantation, the anti-α1,3Gal-independent XAb response is controlled by immunosuppression while the anti-α1,3Gal response is not. This would suggest that XAb responses are T cell-dependent and anti-α1,3Gal Ab responses are not. However, we and other have demonstrated that anti-α1,3Gal, as well as non-α1,3Gal XAb responses are T-cell dependent 28,29.

A second possibility to explain the discrepancy

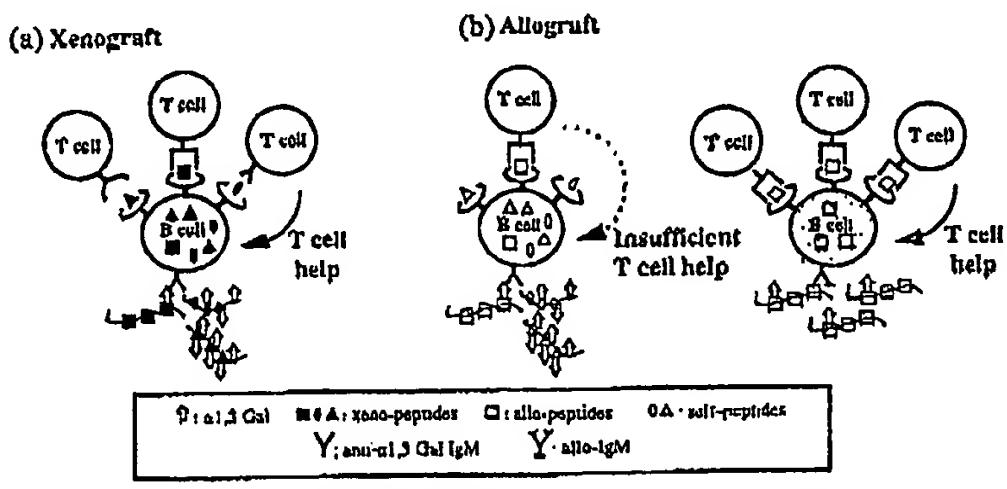


Fig. 6. Model of T-dependent anti-gal Ab production following allograft or xenograft transplantation. Modified from Tanemura et al. 29.

between GT-Ko mice and humans could be that the frequency of B cells producing anti-α1,3Gal Abs is 2–10 per $10^6$ spleen cells in GT-Ko mice (data not shown) but approximately 1% of B cells in peripheral blood in humans 30. High frequency of anti-α1,3Gal B cells could confer resistance to conventional immunosuppression that generally block clonal expansion. The generation of GT-Ko mice with high frequencies of B cells producing anti-α1,3Gal Abs will allow us to test this possibility. Understanding the basis for the differences in the magnitude of anti-Gal Ab responses in GT-Ko mice compared to humans or baboons will direct future manipulation of the GT-Ko mice so that it more accurately models pig-to-human transplantation.

In summary, our studies indicate that GT-Ko mice are able to produce natural as well as elicited anti-α1,3Gal Abs. Anti-α1,3Gal Ab production is more effectively stimulated by xenografts compared to allografts, is restricted to IgM and IgG3 isotypes and is T-cell dependent. Anti-α1,3Gal B cell require cognate interaction with helper T cells, and by-stander activated T cells, such as those providing help for B cells producing alloAbs, cannot provide help for anti-α1,3Gal B cells. Finally, anti-Gal Abs play a minor role in xenograft rejection in this rat-to-GT-Ko mouse model, and is in contrast to the dominant role anti-Gal Abs play in pig-to-baboon or pig-to-human xenotransplantation.

## Acknowledgements

This work was supported by grants from the NIH (AI034061) and Nextran Corp.We thank Drs Janet Plate and Uri Galili for reading of this manuscript and for their helpful comments.

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# The Influence of Genetic Background on Spontaneous and Genetically Engineered Mouse Models of Complex Diseases

Carol Cutler Linder, PhD

**In recent years, the use of genetically altered mice as models of complex human disease has revolutionized biomedical research into the genetics of disease pathogenesis and potential therapeutic interventions. Whether a mouse expresses a spontaneous or induced mutation, it is critical to remember that the observed phenotype is not always the direct result of the genetic alteration. The author points out the importance of considering the genetic background of the strain used to create these important models.**

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The mouse is an ideal model system to tease out the genetics of complex diseases. Random and selective techniques to alter the mouse genome provide powerful tools for biomedical research. Strains carrying specific mutations provide experimental systems for understanding gene function, for studying defects involved in specific human genetic diseases, for preclinical testing of therapeutic agents, and for developing new therapeutic interventions, such as gene therapy[1,2].

The phenotypic characteristics and pathophysiology of mice carrying spontaneous or genetically engineered mutations are most often attributed to alterations in the modified gene. The genetic background and the surrounding environment are often overlooked parameters that can significantly affect the observed phenotype, however. It is extremely important to consider these epigenetic and extragenetic factors when using mice to study complex diseases such as autoimmunity, diabetes, cancer, and cardiovascular disease. The discovery of underlying genes responsible for quantitative traits (those that are affected by more than one gene; *e.g.*, hypertension, hyperglycemia, atherosclerotic lesions, etc.) requires rigorous investigation of how changes in expression of the relevant genes may affect the phenotype of interest. The use of strains with a uniform genetic background greatly facilitates the interpretation of experimental results.

A large number of mouse models are the result of spontaneous single-gene mutations[3]. Traditionally, the detection of spontaneous mutations in an animal colony has been limited to changes in observable phenotypes, such as coat color, growth defects, or alterations in behavior or motor coordination. Large-scale phenotypic screening for desired traits that are not easily observed or measured is time-consuming and not cost-effective, given the rarity of spontaneous mutations. Nevertheless, an important advantage of using a strain with a spontaneous mutation, as opposed to generating a genetically engineered model, is that a researcher can select a mouse model knowing it exhibits certain desired traits and characteristics, whereas a genetically engineered model often does not exhibit the expected phenotype. Positional cloning of the spontaneously mutated gene may lead to the discovery of new pathways for drug intervention (Fig. 1).

## Genetic Engineering Technologies

Three broad areas of technology—transgenesis, targeted mutagenesis using homologous recombination, and random mutagenesis—are currently used to create genetically engineered strains of mice.

Transgenic mice have genetic material randomly added to their genomes[4]. Such strains have been used to study gene function and expression and as a result have become important disease models. Since transgene insertion is a random event, the phenotype of the mouse may vary, depending on the site of integration and the copy number of transgenes integrated. Transgene integration may cause disruption in an endogenous gene, creating an inherited phenotype (usually recessive) unrelated to transgene expression. In these

cases, the transgenic animal provides a vehicle for gene discovery through the mapping and subsequent cloning of a disrupted gene.

In targeted mutagenesis, homologous recombination alters or replaces a specific locus or gene[5,6]. Currently, the majority of strains created by gene targeting carry a null mutation (*i.e.*, "knockout") for the gene in question. More recently created conditional targeted mutations allow control of the tissue specificity of the mutation or onset of gene expression (temporal control)[7,8]. Gene targeting produces strains used to study gene function and to create models for human genetic diseases for which the offending gene is known.

Transgenesis and targeted mutagenesis technologies often produce unexpected results, creating mice with either no observable change in phenotype or an unexpected phenotype that may be outside the researcher's area of expertise or interest. This gene-based approach may, however, lead to the discovery of novel pathways of an already known gene (Fig. 1).

Random mutagenesis protocols such as treating mouse gametes or ES cells with chemical mutagens[9] and gene trapping with retroviral vectors[10] are also used to produce valuable new models. These random approaches produce both dominant and recessive mutations, although most efforts to date have concentrated on the more easily identified dominant mutations. Like the use of spontaneous mutations, random mutagenesis is a phenotype-driven, rather than gene-driven, approach. To obtain maximum value from random mutagenesis approaches, rapid and systematized protocols for phenotypic screening, as well as sufficient resources for mapping and cloning genes and subsequently distributing these new models must be available. Several large-scale mutagenesis projects currently underway employ the alkylating agent ethylnitrosourea (ENU) to induce random mutations in the mouse genome[11,12]. In addition, government agencies are currently funding ENU mutagenesis initiatives in a wide variety of disciplines[13].

## Factors Affecting Phenotype

The expression of a phenotype in mice carrying a spontaneous or induced mutation may depend on a number of factors not readily apparent to the initial researcher, nor to those using the model in subsequent studies. Environment and genetic background are two major contributors to phenotype. Other factors include mutations that are actually hypomorphs (*i.e.*, mutations that cause only a partial decrease in gene expression) rather than null alleles; compensatory pathways; and transgenesis-specific factors, including site of integration, transgene copy number, and insertional mutations.

The health status of a colony may alter the observable phenotypes, especially for immunocompromised strains. For example, severe ulcerative colitis in T-cell receptor alpha- and T-cell receptor beta- deficient mice[14] virtually disappeared following rederivation into the Induced Mutant Resource colony at The Jackson Laboratory. Both interleukin-2 (IL2)- and interleukin-10 (IL10)- deficient mice demonstrated similar findings regarding the effect of health status on the display of disease signs[15-18].

"Genetic background" is defined as a collection of all genes present in an organism that influence a trait or traits. While most of the commonly used inbred strains share a fairly common origin[19], each strain has its own unique set of characteristics or background lesions[20] (Table 1). Strain characteristics are the result of sequence differences in genes (allelic variance) among inbred strains. Strain attributes can be the result of single genes (*e.g.*, the retinal degeneration 1 mutation that causes blindness in inbred strains like C3H/HeJ, FVB/N, and SJL/J[3]) or a combination of genes (*e.g.*, differential susceptibilities of inbred strains to diet-induced obesity[21]).

The phenotype of mice carrying a modified gene will vary depending on the genetic background because of the pres-

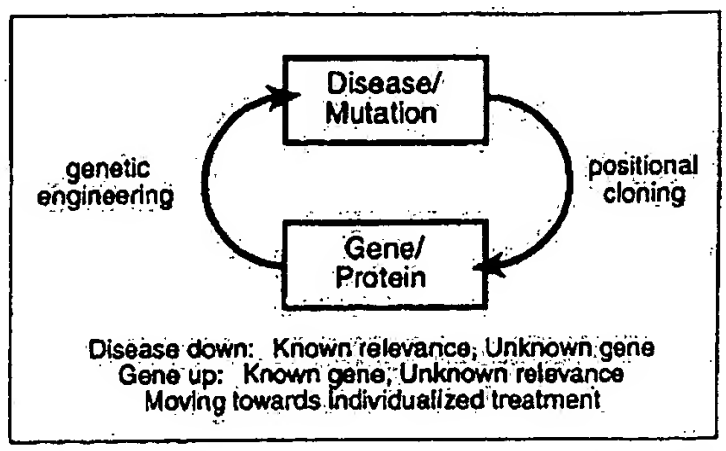


**FIGURE 1. Gene discovery and models. Mouse models for human disease arise from two general approaches: 1) a mouse exhibits a phenotype, caused by a spontaneous or randomly induced (*e.g.*, radiation or ENU) mutation, that mimics a human condition or disease. Positional cloning of the causative gene(s) may lead to the discovery of a gene or pathway not previously implicated as important to the disease state; 2) a gene is implicated, through a variety of mechanisms (*e.g.*, known human mutations, cell-based assays, etc.), to be involved in a human condition or disease. Genetic engineering techniques, such as transgenesis or targeted mutagenesis, are used to manipulate this gene in the mouse. The resulting phenotypes of mice that either overexpress or lack a functional protein are often unexpected and may suggest a previously unidentified function for that gene. Both approaches may lead to the discovery of novel *in vivo* pathways for drug discovery and disease intervention.**

**TABLE 1. Inbred strain characteristics.**

| Characteristic | DBA/2J | C57BL/6J |
|---|---|---|
| Audiogenic seizures | Susceptible | Resistant |
| Eye defects | Develop hereditary glaucoma | High incidence of microphthalmia |
| Diet-induced atherosclerosis | Low susceptibility | High susceptibility |
| Response to alcohol and morphine | Extreme intolerance | High preference |

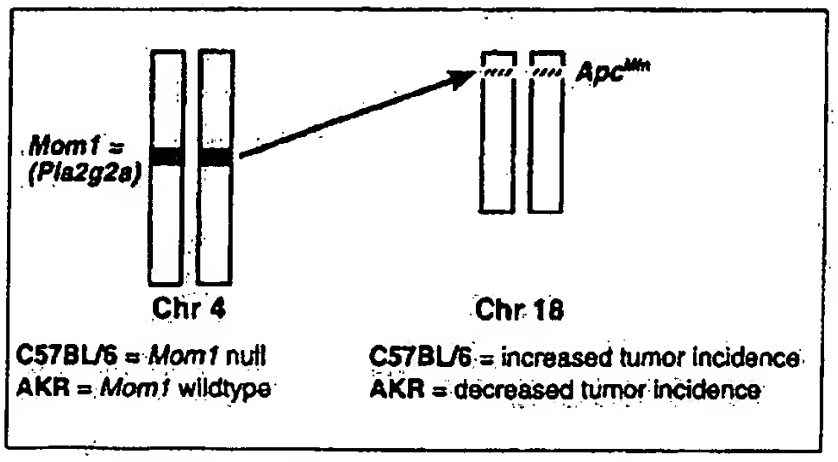


**FIGURE 2. Phenotypic effects of unlinked modifier genes. Tumor incidence in mice heterozygous for the *Apc^Min^* mutation varies depending on genetic background. *Mom 1* (modifier of *Min*) was mapped to Chromosome 4 and later identified to be the result of a mutation in the secretory type II phospholipase 2A gene (*Pla2g2a*). Although not the only genetic modifier, *Pla2g2a* confers resistance to intestinal tumorigenesis. C57BL/6J mice are homozygous for a null allele of *Pla2g2a* and thus display an increased tumor incidence compared to AKR mice, which are wildtype for *Pla2g2a*.**

ence of genetic modifiers (allelic variants at loci other than the one being genetically modified) in the inbred strain genome. Genetic modifiers may function through a number of mechanisms: (1) they can suppress or enhance the expression of genes involved in physiological or pathological pathways; (2) they can alter DNA transcription rates or mRNA stability; (3) they can have epigenetic effects causing changes in DNA methylation or chromatin structure; and (4) they can result from a variation of gene copy number[22].

Many of the classic spontaneous mutations arose on a mixed or undefined genetic background. Induced mutations (both targeted and random) and transgenics are often either generated or initially maintained on a mixed segregating or hybrid background (*e.g.*, C57BL/6 and 129 for targeted mutations or C57BL/6 and SJL hybrid for transgenics). Further inbreeding or backcrossing to other inbred strains may result in a phenotype significantly different from that initially reported on the mixed genetic background. Interpreting such phenotypic differences is often difficult, and it is essential to use the appropriate controls.

As discussed, phenotypic differences among different strains carrying the same mutation may be the direct result of genetic modifiers that are unlinked to the modified gene. For example, tumor incidence of mice heterozygous for the *Apc^Min^* chemically induced mutation varies depending on the genetic background (Fig. 2). *Mom1* (modifier of *Min*) was mapped to Chromosome 4 (Chr 4) and later identified to be the result of a mutation in the secretory type II phospholipase 2A gene (*Pla2g2a*). Although not the only genetic modifier, *Pla2g2a* confers resistance to intestinal tumorigenesis[23,24]. C57BL/6J mice are homozygous for a null allele of *Pla2g2a*, and thus display an increased tumor incidence compared to AKR mice, which are wildtype for *Pla2g2a*.

Alternatively, the observed phenotype may be due to genes completely independent of the modified gene or transgene, as Fig. 3 illustrates. SJL/J mice are homozygous for the retinal degeneration 1 mutation (*Pde6b^rd1^*), which causes blindness by age of weaning. C57BL/6J mice are wildtype at this locus. B6SJLF1 mice are sighted because *Pde6b^rd1^* is inherited recessively. Backcrossing mice hemizygous for a transgene (Tg/0) to a B6SJLF1 hybrid (*Pde6b^rd1^*/+) is a common breeding scheme for transgenic mice. Fifty percent of the progeny will be carrying the transgene and 25% of the progeny will be homozygous for the retinal degeneration 1 mutation. The transgene and *Pde6b^rd1^* mutation will segregate independently in progeny unless the transgene has integrated into the genome in a location that is closely linked to the *Pde6b^rd1^* mutation. Therefore, if characterization of transgenic mice requires mice to see normally, it is important to genotype mice for both the presence of the transgene and the absence of *Pde6b^rd1^* in the homozygous state.

A linked gene (carried over during backcrossing to produce a congenic strain), rather than the modified gene, may also be responsible for phenotypic differences between mutant mice and their

**TABLE 2. Effect of genetic background on interleukin-2 (IL2) targeted mutations ("knockouts").**

| Strain Name | Genetic Background | Phenotype |
|---|---|---|
| B6;129-*Il2^tm1Hor^* | Segregating mixture of C57BL/6 and 129P2/OlaHsd genomes | • 50% die between 4 and 9 weeks of age<br>• Splenomegaly, lymphadenopathy, severe anemia<br>• Progressive inflammatory bowel disease (IBD) |
| B6.129P2-*Il2^tm1Hor^* | C57BL/6 congenic (N10) | • Generalized autoimmune disease (hemolytic anemia)<br>• Die by 3-6 months of age<br>• Progressive IBD, dependent on health status |
| C3.129P2(B6)-*Il2^tm1Hor^* | C3H/HeJ congenic (N10) | • Generalized autoimmune disease (hemolytic anemia)<br>• Die by 7 weeks of age |
| C.129P2(B6)-*Il2^tm1Hor^* | BALB/c congenic (N10) | • Generalized autoimmune disease (hemolytic anemia)<br>• Die by 7 weeks of age |

inbred strain controls, as illustrated in Fig. 4. The apolipoprotein E targeted mutation (*Apoe*$^{tm1Unc}$) was generated in a 129P2-derived embryonic stem (ES) cell line[25]. 129P2 mice are homozygous for both the pink-eyed dilution (*p*) and chinchilla mutations (*Tyr*$^{c-ch}$), which, like *Apoe*, are located on Chr 7. The *Apoe*-targeted mutation was backcrossed to C57BL/6J mice for further characterization. A homozygous colony was generated after six backcross generations. Mice from this N6 colony were silver with pink eyes because the pink-eyed dilution gene remained linked to *Apoe*$^{tm1Unc}$. Further backcrossing to N10 reduced the linked segment through crossover events and congenic mice are now the expected black coat color with dark eyes.

## Phenotypes Affected by Genetic Background

There are many well-characterized instances of the influence of inbred background on the expressed phenotype. For example, on a mixed C57BL/6J-SJL inbred background, an activated HRAS transgene under the control of the whey acidic protein promoter results in mammary and salivary carcinomas that frequently metastasize to the lung. On the FVB inbred background, this transgene causes anaplastic carcinomas that do not metastasize[26]. Tumor type and onset in mice lacking p53 (*Trp53*) is also dependent on the strain background[27].

Genetic engineering technologies have allowed researchers to examine the importance of individual cytokines in autoimmune diseases, such as inflammatory bowel syndrome, rheumatoid arthritis, and systemic lupus erythematosus. Interestingly, mice lacking IL2 display a number of autoimmune-related defects that vary in both severity and onset depending on genetic background (Table 2)[16,28,29].

Inbred strains also vary in their genetic predisposition to diabetes, a classic example of which are spontaneous mutations in the leptin signaling pathway. The original diabetes mutation (*Lepr*$^{db}$) arose spontaneously in the C57BLKS strain. Homozygous mutant mice (*db/db*) show extreme obesity and rapidly develop sustained and overt diabetes, characterized by hyperglycemia and hyperinsulinemia[30]. The obese mutation (*Lep*$^{ob}$) occurred spontaneously in a stock carrying multiple recessive mutations and was backcrossed to C57BL/6 for characterization[31]. The phenotype of obese homozygous mutant mice (*ob/ob*) is very similar to the *db/db* mice. However, the pancreatic beta cells in C57BL/6 *ob/ob* mice can compensate for the lack of leptin, and overt diabetes is only transient. *ob/ob* mice on the C57BLKS background result in a phenotype that is indistinguishable from that of *db/db*. Likewise, *db/db* mice show only transient diabetes on the C57BL/6 background[32].

Interpreting phenotypic information in complex disease models on a mixed segregating genetic background can be tricky. The targeted disruption of the insulin receptor (*Insr*) gene illustrates this problem. Neonatal lethality due to severe diabetes is observed in homozygous null mice[33]. Variable hyperinsulinemia is observed in heterozygous mice on a mixed segregating background, indicating phenotypic interference by undefined background modifiers. On a C57BL/6 congenic background, heterozygous insulin receptor knockouts showed only mild hyperinsulinemia; in contrast, heterozyotes on a 129S6 congenic background showed severe hyperinsulinemia and insulin resistance[34]. Five quantitative trait loci (QTL) contributing to the phenotype were identified in progeny of an intercross between C57BL/6 and 129S6 congenic stocks. Of these, four deleterious QTLs were contributed by the seemingly hyperinsulinemia-resistant C57BL/6 background, with only one deleterious QTL identified from the hyperinsulinemia-permissive 129S6 background[34].

Both genetic and environmental factors contribute to the complex disease of essential hypertension (*i.e.*, high blood pressure without identifiable cause). Both the natriuretic peptide and the renin/angiotensin systems regulate blood pressure. These pathways have been dissected using genetic

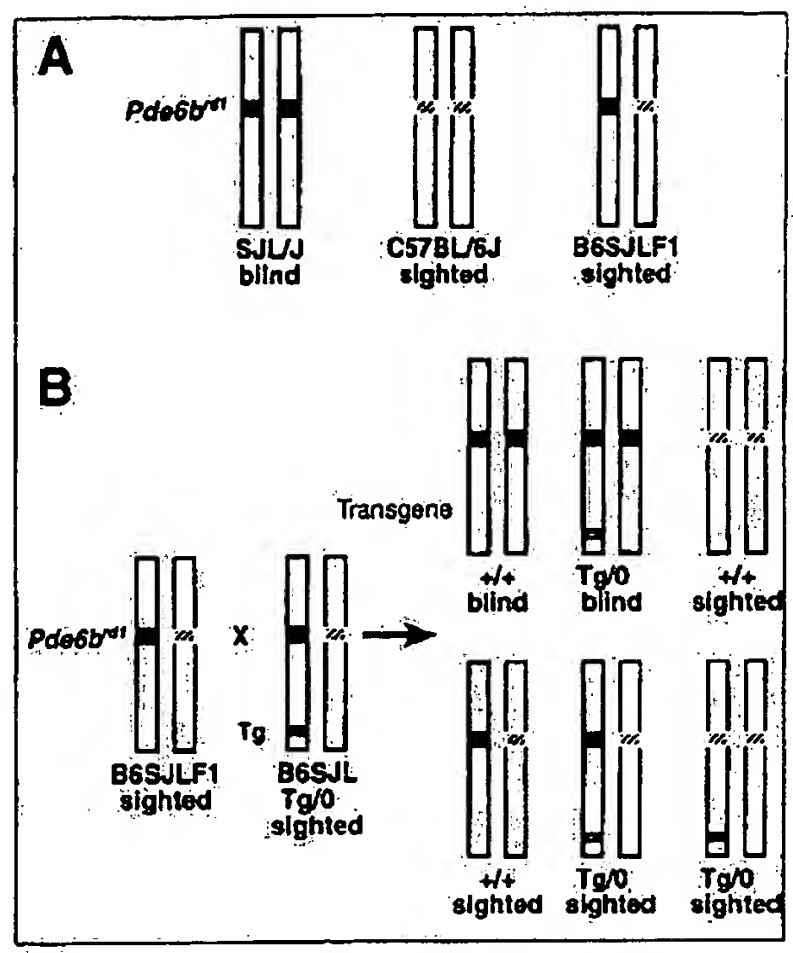


**FIGURE 3. Phenotypic effects of genes independent of transgene. A. SJL/J mice are homozygous for the retinal degeneration 1 mutation (*Pde6b*$^{rd1}$), which causes blindness by wean age. C57BL/6J mice are wildtype at this locus. B6SJLF1 mice are sighted because *Pde6b*$^{rd1}$ is inherited recessively. B. Backcrossing mice hemizygous for a transgene (Tg/0) to a B6SJLF1 hybrid (*Pde6b*$^{rd1}$/+) is a common breeding scheme for transgenic mice. 50% of the progeny will be carrying the transgene and 25% of the progeny will be homozygous for the retinal degeneration 1 mutation. The transgene and *Pde6b*$^{rd1}$ mutation will segregate independently in progeny unless the transgene has integrated into the genome in a location that is closely linked to the *Pde6b*$^{rd1}$ mutation. Therefore, if characterization of transgenic mice requires mice to see normally, it is important to genotype mice for both the presence of the transgene and the absence of *Pde6b*$^{rd1}$ in the homozygous state.**

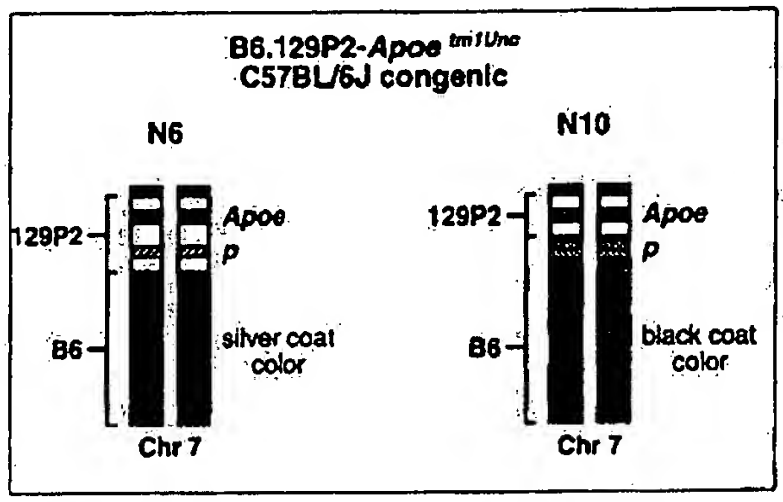


**FIGURE 4. Phenotypic effects of linked genes carried over during backcrossing. The apolipoprotein E targeted mutation (*Apoe*$^{tm1Unc}$) was generated in an 129P2-derived ES cell line. 129P2 mice are homozygous for both the pink-eyed dilution (*p*) and chinchilla mutations (*Tyr*$^{c-ch}$), which, like *Apoe*, are located on Chr 7. The *Apoe* targeted mutation was backcrossed to C57BL/6J mice for further characterization. A homozygous colony was generated after six backcross generations. Mice from this N6 colony were silver with pink eyes because the pink-eyed dilution gene remained linked to *Apoe*$^{tm1Unc}$. Further backcrossing to N10 reduced the linked segment through crossover events and congenic mice are now the expected black coat color with dark eyes.**

manipulation of a number of genes within these pathways[35-37]. Proper interpretation of results requires close attention to the genetic background carrying the modified gene. Interestingly, C57BL/6 and the 129 strains actually differ in renin gene copy number[38]. C57BL/6 mice have one copy per haploid genome, while 129 strains naturally have two copies. Typing of the renin gene copy number in pro-atrial natriuretic peptide- (proANP) deficient mice on a mixed C57BL/6-129 genetic background suggests that blood pressure varies according to gene copy number.

## Recommendations

The selection of models for the study of complex diseases may be difficult and the choices available to the researcher are not always ideal. Often ignored or poorly understood, genetic background contributes significantly to the phenotype of mouse models in a wide range of disciplines. Whether generating models or using mice already produced by another researcher, the following recommendations should assist in the proper interpretation of experimental results.

Whenever possible, select both the optimal genetic background as well as the gene of interest. Analysis of a mutation on four or five different inbred backgrounds may lead to the discovery of modifier genes key to the understanding of human disease. This can be accomplished either by creating genetically engineered mice on a standard, well-characterized inbred background (thereby achieving isogenicity with an inbred strain)[39,40], or by generating congenic strains through traditional[40] or marker-assisted protocols[41,42] (thereby achieving congenicity).

In some instances, it is impossible to avoid the use of a mixed segregating or hybrid background (*e.g.*, because of embryonic lethality on an inbred background). The use of wildtype controls and an understanding of the impact of genes segregating independently of a modified gene is essential. Finally, it is important to be cognizant of environmental factors, ranging from colony health status to diet and light cycle, which may affect the phenotype of the mouse.

*Received 3/28/01; accepted 4/11/01.*

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5 September 1997; accepted 14 November 1997

# Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

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Ovine primary fetal fibroblasts were cotransfected with a neomycin resistance marker gene (*neo*) and a human coagulation factor IX genomic construct designed for expression of the encoded protein in sheep milk. Two cloned transfectants and a population of neomycin (G418)–resistant cells were used as donors for nuclear transfer to enucleated oocytes. Six transgenic lambs were liveborn: Three produced from cloned cells contained factor IX and *neo* transgenes, whereas three produced from the uncloned population contained the marker gene only. Somatic cells can therefore be subjected to genetic manipulation in vitro and produce viable animals by nuclear transfer. Production of transgenic sheep by nuclear transfer requires fewer than half the animals needed for pronuclear microinjection.

Microinjection of DNA into the pronuclei of fertilized oocytes has been the only practical means of producing transgenic livestock since the method was established in 1985 (*1*). However, only a small proportion (~5%) of animals integrate the transgene DNA into their genome (*2*, *3*). In addition, because the timing and site of integration are random, many transgenic lines do not provide sufficiently high levels of transgene expression or germline transmission. The consequent inefficient use of animals and associated high costs are a major drawback to pronuclear microinjection.

In mice, embryonic stem cells provide an alternative to pronuclear microinjection as a means of transferring exogenous DNA to the germline of an animal and allow precise genetic modifications by gene targeting (*4*, *5*). However, despite considerable efforts, embryonic stem cells capable of contributing to the germline of any livestock species have not been isolated (*6*–*11*).

Recently, viable sheep have been produced by transfer of nuclei from a variety of somatic cell types cultured in vitro (*12*–*14*). We now demonstrate that nuclear transfer from stably transfected somatic cells provides a cell-mediated method for producing transgenic livestock.

We have used a transgene designed to express human clotting factor IX (FIX) protein in the milk of sheep. FIX plays an essential role in blood coagulation, and its deficiency results in hemophilia B (*15*). This disease is currently treated with FIX derived mainly from human plasma. Recombinant FIX produced in milk would provide an alternative source at lower cost and free of the potential infectious risks associated with products derived from human blood.

The transgene construct, pMIX1 (*16*), comprises the human FIX gene, containing the entire coding region (*17*), linked to the ovine β-lactoglobulin (BLG) gene promoter, which has been previously shown to provide a high level of transgene expression in ovine mammary glands (*18*). Analysis of pMIX1 expression in transgenic mice showed that seven of seven female founders expressed FIX in their milk (*19*). The level of expression in two animals (125 μg/ml) exceeded that achieved in previous studies (*20*, *21*), indicating that pMIX1 is functional and suitable for introduction into sheep.

Primary strains of ovine cells, termed PDFF (Poll Dorset fetal fibroblast) 1 to 7, were derived from seven day-35 fetuses from the specific pathogen-free flock at PPL Therapeutics (*22*). Sex analysis of each cell strain by the polymerase chain reaction (PCR) (*23*) revealed PDFF5 to be male and

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**Table 1.** Results of nuclear transfer. Nuclear transfer was performed as described previously (*12*, *13*). All cells were exposed to a reduced serum concentration (0.5%) for 5 days before use as nuclear donors. PDFF5 cells were used for nuclear transfer at passage 2 or 3, PDFF2 transfected pools at passage 5 to 7, and transfected clones PDFF2-12 and PDFF-13 at passage 7 to 9. Liveborn lambs were defined as those with a heartbeat and able to breathe unassisted at birth.

| Measurement | PDFF5 (non-transfected) | PDFF2 pool | PDFF2-12 | PDFF2-13 |
|---|---|---|---|---|
| Reconstructed embryos | 82 | 224 | 89 | 112 |
| No. developed to morulae or blastocysts | 5 (6.1%) | 22 (9.8%) | 19 (21.4%) | 23 (20.5%) |
| Embryos transferred | 5 | 22 | 19 | 21 |
| Recipients | 2 | 9 | 7 | 6 |
| Pregnancies at day 60 | 2 | 4 | 4 | 1 |
| Fetuses at day 60 (% of embryos transferred) | 3 (60%) | 4 (18.2%) | 6 (31.6%) | 1 (4.8%) |
| Liveborn lambs (% of embryos transferred) | 1 (20%) | 3 (13.6%) | 2 (10.5%) | 1 (4.8%) |
| Nuclear transfer efficiency (% live lambs from reconstructed embryos) | 1.22% | 1.34% | 2.25% | 0.89% |

the other six to be female.

Trial experiments indicated that both PDFF2 and PDFF5 cells could be readily transfected with a *lacZ* reporter gene with the use of the cationic lipid reagent Lipofectamine. PDFF2 cells at passage 1, after 3 days in culture, were cotransfected with pMIX1 DNA and the selectable marker construct PGKneo, and stable transfectants were selected with G418. Because the effects of drug selection and growth as single-cell clones on the ability of cells to support nuclear transfer were unknown, cells were then treated in two ways: One group was grown at high density under G418 selection and then cryopreserved as a pool for nuclear transfer. The other group was plated at low density under G418 selection, and cloned transfectants were grown from isolated colonies (*22*). A total of 24 clones was isolated, of which 21 were expanded for analysis of genomic DNA. Ten clones were found to contain pMIX1 by DNA hybridization analysis (*24*).

Untransfected PDFF2 cells cultured to passage 19 over a period of 80 days exhibited a modal chromosome number of 54, the euploid ovine chromosomal complement. The chromosome number of the four most rapidly growing pMIX1-transfected clones (PDFF2-12, -13, -31, -38) was determined at passage 6 or 7, after an average of 40 days in culture, and that of the uncloned PDFF2 pool was determined at passage 5, after 19 days in culture. Each clone and the pool showed a modal chromosome number of 54, indicating the absence of gross chromosomal instability during culture and drug selection.

We have proposed that induction of quiescence in nuclear donor cells by serum deprivation is necessary for successful nuclear transfer (*12*). After 5 days of culture in medium with a reduced serum content (0.5%), immunofluorescence detection of proliferating-cell nuclear antigen (PCNA), which is an indicator of active DNA replication, showed that none of the cells analyzed was in S phase, consistent with cell cycle arrest (*25*). Restoration of serum content to 10% reversed this effect and cell growth resumed.

Four cell types were used as nuclear donors: untransfected male PDFF5 cells, pooled female PDFF2 transfectants, and two transfected clones, PDFF2-12 and PDFF2-13, which contained >10 and ~5 copies of the pMIX1 transgene, respectively. Transfer of nuclei from each cell type into enucleated oocytes derived from Scottish Blackface ewes was performed as previously described (*12*, *13*).

Live lambs were obtained from all four cell types (Table 1). As expected, animals derived from PDFF5 cells were male and those from PDFF2 cells were female. The efficiency of nuclear transfer, expressed as the number of liveborn lambs obtained per 100 reconstructed embryos, varied from 0.89% for PDFF2-13 to 2.25% for PDFF2-12. This efficiency is similar to the value (1.35%) that we obtained previously for nonmanipulated fetal fibroblasts from another breed of sheep (BLWF1) (*13*).

Pregnancies resulting from embryo trans-

**Table 2.** Characteristics of nuclear transfer–derived lambs. Outcomes of 11 pregnancies resulting from nuclear transfer of PDFF donor cells. When judged necessary, labor was induced by injection of dexamethasone at day 153 of gestation; when required, cesarean section (CS) was performed 24 to 52 hours later. The average duration of gestation for the Poll Dorset flock at PPL Therapeutics is 145 days.

| Pregnancy no. | Nuclear transfer donor cell type | Lamb | Gestation (days) | Birth weight (kg) | *neo* | FIX | Sex | Comments |
|---|---|---|---|---|---|---|---|---|
| 1 | PDFF5 | 7LL5 | 147 | 3.8 | | | M | Unassisted birth |
| 2 (twins) | PDFF5 | 7LL6* | 150 | 3.4 | | | M | Stillbirth, one fetus dead for ≤1 week |
| | | 7LL7* | 150 | 3.7 | | | M | |
| 3 | PDFF2 pool | | <80 | | | | | Regressed |
| 4 | PDFF2 pool | 7LL8 | 155 | 7.6 | (+) | (−) | F | Assisted birth because of position of lamb |
| 5 | PDFF2 pool | 7LL9* | 161 | 6.3 | (+) | (−) | F | Induced, CS 52 hours later, died 90 min postpartum, meconium in lung |
| 6 | PDFF2 pool | 7LL12 | 155 | 8.7 | (+) | (−) | F | Induced, CS 52 hours later |
| 7 | PDFF2-12 | 7LL3* | 130 | | | | F | Spontaneous abortion |
| 8 (twins) | PDFF2-12 | 7LL10* | 132 | 3.6 | (+) | (+) | F | Loss of fetal heartbeat, induced, CS, |
| | | 7LL11* | 132 | 4.5 | (+) | (+) | F | stillbirth, one fetus abnormal |
| 9 | PDFF2-12 | 7LL14* | 148 | 3.6 | (+) | (+) | F | Induced, CS 24 hours later, heartbeat, no breathing |
| 10 (twins) | PDFF2-12 | 7LL15 | 155 | 4.6 | (+) | (+) | F | Induced, unassisted birth, 7LL16 |
| | | 7LL16* | 155 | 3.0 | (+) | (+) | F | euthanized at 14 days, heart defect |
| 11 | PDFF2-13 | 7LL13 | 155 | 5.5 | (+) | (+) | F | Induced, unassisted birth |

*Lamb died or was euthanized for animal welfare reasons.

fer were determined by ultrasound scan at about 60 days after estrus, and development was subsequently monitored at regular intervals. Of the original 14 fetuses, 7 were liveborn, as defined by heartbeat and unassisted breathing (Table 2). Postmortem examination of aborted fetuses and dead lambs did not reveal any common factor as a cause of death.

All animals derived from PDFF cells exhibited a prolonged gestation, and, with the exception of animals 7LL5 to 7LL8, labor was induced artificially. Delayed delivery was likely the cause of death of lamb 7LL9. Subsequently, all surrogate ewes were induced at day 153, and, if necessary, cesarean section was performed. Three of 11 pregnancies were twin pregnancies. In two instances (7LL6 and 7LL7 and 7LL10 and 7LL11), the death of one fetus in late pregnancy probably resulted in the death of the sibling.

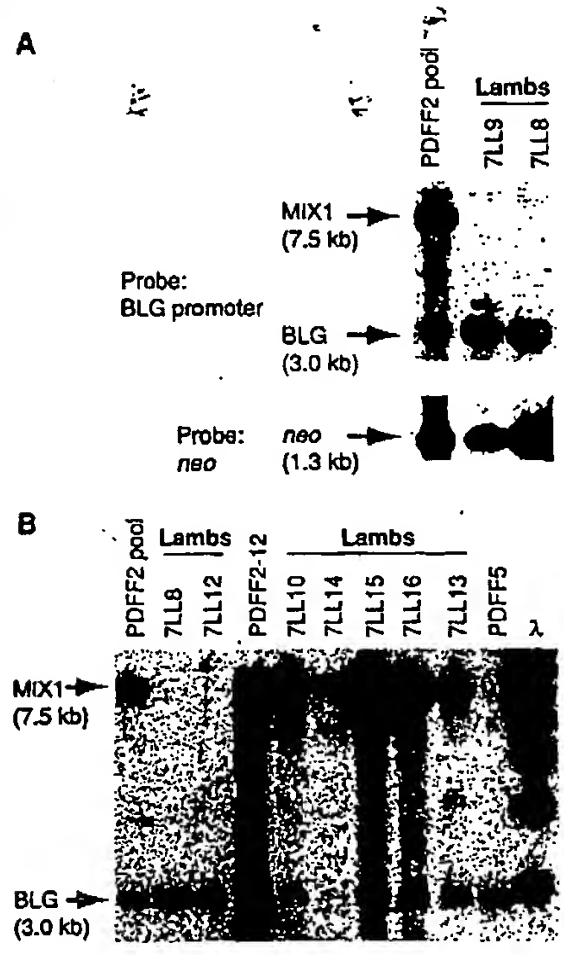


**Fig. 1.** DNA analysis of transfected clones and transgenic sheep. Genomic DNA was isolated from the blood of live animals or tongue samples from dead animals, digested with Bam HI and Eco RI, and subjected to Southern hybridization with either a 1.8-kb fragment of the BLG promoter or the *neo* gene. (**A**) Southern analysis of the uncloned pool of cells (PDFF2 pool), and two lambs (7LL8 and 7LL9) derived from them, for the presence of pMIX1 and PGKneo. (**B**) Assay for the presence of the pMIX1 transgene in lambs derived from the PDFF2 pool (7LL8 and 7LL12) and from the transfected clones PDFF2-12 (7LL10, 7LL14 to 7LL16) and PDFF2-13 (7LL13). PDFF5 cells were not transfected. The positions and sizes of fragments corresponding to the transgenes and the endogenous BLG gene are indicated. The lane marked λ is a 1-kb ladder of phage λ fragments from 3 to 12 kb.

The birth weight of nuclear transfer–derived lambs whose gestation exceeded 145 days ranged from 3.0 to 8.7 kg, with a mean of 3.7 kg for twins and 5.9 kg for single pregnancies. Poll Dorset lambs in the PPL Therapeutics New Zealand–derived flock have mean weights of 3.75 kg for twins and 5.1 kg for single pregnancies. However, comparison is complicated by the fact that nuclear transfer–derived lambs were gestated in Scottish Blackface surrogate mothers. All animals from PDFF2 cells had an undershot lower jaw that did not interfere with their well-being. This characteristic is a genetic trait that occurs sporadically in the Poll Dorset breed and is considered to be unrelated to nuclear transfer. The PDFF5 lambs did not show this feature.

DNA from nuclear transfer–derived lambs was analyzed for the presence of pMIX1 and PGKneo transgenes (Fig. 1). All fetuses and animals derived from the transfected PDFF2 cells were transgenic. The three animals derived from the PDFF2 pool (7LL8, -9, -12) contained the selectable marker gene but lacked the FIX transgene (Fig. 1, A and B). Fetuses and lambs derived from the cell clones PDFF2-12 (7LL10, -14, -15, -16) and PDFF2-13 (7LL13) contained both the FIX transgene (Fig. 1B) and PGKneo.

Our approach has shown that cell-mediated transgenesis is possible in a mammal other than the mouse. The technique is still in the early stages of development and problems remain to be addressed—in particular, the lack of spontaneous parturition and the incidence of perinatal mortality. However, the mortality rate we observed (46%) was exacerbated by two twin pregnancies in which the death of one lamb in late gestation may have resulted in the loss of the other. The mortality rate for nontwin pregnancies was 28.6%, higher than that occurring after normal breeding (~8%) but similar to that observed after nuclear transfer with embryonic blastomeres (5 to 40%) (*26*). Our data therefore do not suggest any correlation between lamb mortality and extended culture or genetic manipulation of the donor cell. Many types of manipulation of preimplantation embryos—for example, in vitro oocyte maturation and fertilization, in vitro culture, asynchronous embryo transfer, and progesterone treatment of the mother—have been shown to increase fetal morbidity and mortality (*26*, *27*). An increased understanding of the interaction between the transplanted nucleus and the host cytoplasm and the relation between the early embryo and the maternal environment, together with improved culture systems, should increase the success of embryo production and manipulation in vitro.

The use of somatic cell donors for nuclear transfer in livestock offers many advantages over pronuclear microinjection. Since 1989, PPL Therapeutics has generated a substantial number of transgenic sheep by pronuclear microinjection. A total of 51.4 animals are required to produce one transgenic lamb by pronuclear microinjection, compared with 20.8 animals in the present study by nuclear transfer, values that differ by a factor of ~2.5 (Table 3). The most important difference is that no recipients are wasted gestating nontransgenic lambs in the nuclear transfer technique.

Gestation of large numbers of nontransgenic embryos represents a major source of inefficiency (*28*). Several schemes have been devised to identify transgenic embryos before embryo transfer, either by detection of the transgene in embryo biopsies by PCR (*29*) or by co-expression of a marker gene (*30*, *31*). However, these methods, with the possible exception of that of Takada *et al.* (*30*), are restricted by the persistence of

**Table 3.** Comparison of the production of transgenic sheep by nuclear transfer or pronuclear microinjection.

| Parameter | Pronuclear microinjection (1989–1996) | Nuclear transfer of PDFF2 transfectants |
|---|---|---|
| Oocyte donors | 982 | 68 |
| Intermediate recipients* | Not applicable | 14 |
| Final recipients | 1895 | 22 |
| Total number of sheep used | 2877 | 104 |
| Established pregnancies (% of final recipients) | 912 (48%) | 9 (41%) |
| Lambs born | 1286 | 6 |
| Viable transgenic lambs born† | 56 | 5 |
| Percentage of offspring transgenic | 4.35% | 100% |
| Sheep required for production of one transgenic lamb | 51.4 | 20.8 |

*After nuclear transfer, intermediate recipients are used to allow development of reconstructed embryos to blastocyst stage. †Defined as those alive at 1 week of age.

unintegrated DNA during the short time that embryos can be cultured before embryo transfer. In contrast, cells transfected in vitro can be analyzed extensively before effort is devoted to large animals. This advantage will be particularly important in instances in which microinjection is inefficient; for example, with large constructs such as yeast artificial chromosomes.

Delayed integration of microinjected DNA into the embryo genome often results in mosaic founder animals. The reduced rate of transgene transmission resulting from germline mosaicism can hinder or prevent the establishment of transgenic lines from potentially valuable founder animals. In contrast, animals produced by nuclear transfer are entirely transgenic.

Nuclear transfer allows the sex of transgenic animals to be predetermined and thus offers a further twofold increase in efficiency relative to pronuclear microinjection when the sex of the transgenic founder animal is critical. If, for example, the primary interest is the expression of human proteins in milk, the founder generation can be all females. Sheep with different random integrations of the transgene can be produced by nuclear transfer from independent cell clones and the milk analyzed. After a suitable clone has been identified, the corresponding stock of cells can be used to generate an "instant flock" by further nuclear transfer. Such a flock could be superior to those produced by conventional breeding as a source of proteins for human therapy because genetic identity would contribute to the consistency of the medicinal product.

The procedures of transfection, drug selection, and growth from single-cell clones described here are essentially the same as those required for gene targeting. The realistic prospect of targeted genetic manipulation in a livestock species should open a vast range of new applications and research possibilities.

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16. For construction of pMIX1, human FIX DNA from an Nhe I site at position 2982 to a Bst BI site at position 33,840 (positions refer to EMBL database file HSFIXG) was derived from overlapping genomic λ clones. A 3′ fragment from the Bst BI site to position 34,346 was derived by PCR amplification and a Xho I site introduced. The 31.36-kb fragment containing the FIX coding region was assembled in Bluescript KS− vector (Stratagene) that had been digested with Xba I and Xho I. The entire FIX gene was then excised as a Not I–Xho I fragment and inserted into the ovine BLG expression vector pMADS+ that had been digested with Not I and Xho I. The pMADS+ vector consists of 4.2 kb of BLG promoter sequence, 30 bp of 5′ untranscribed region, a multicloning site (Eco RV, Not I, Bgl II, Nhe I, Xho I, Cla I), 170 bp of 3′ untranscribed region including a polyadenylate addition site, and 2 kb of 3′ flanking region cloned into pUC18, containing a modified polylinker to allow excision of the insert with either Mlu I or Pvu I.
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19. Transgenic mice were produced by microinjection of a 37.8-kb Mlu I fragment of pMIX1 into the male pronucleus of fertilized mouse oocytes. Milk was collected from transgenic founder females at day 10 postpartum. FIX protein in milk was assayed by enzyme-linked immunosorbent assay with polyclonal rabbit antiserum to human FIX (Dako) and quantified by comparison with purified human FIX standard (Diagnostica Stago).
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22. PDFF cell strains were isolated by disaggregation of day-35 fetuses and cultured as described previously (*13*). At first passage, 2 × $10^5$ PDFF2 cells were plated into a 3.5-cm-diameter well and cotransfected with 0.5 μg of PGKneo and 1.5 μg of Mlu I–digested pMIX1 DNA with the use of Lipofectamine (Gibco). Forty-eight hours after transfection, cells were split 1:10 and G418 added to a final concentration of 0.6 mg/ml. Transfected PDFF2 cells achieved subconfluence after selection for 6 days. At the third passage, one portion of the cells was split 1:10 and then subjected to selection for an additional 5 days before cryopreservation as an uncloned population. Other portions were split 1:1000 or 1:5000 and subjected to G418 selection for an additional 7 days. Individual colonies were isolated and expanded for cryopreservation at passage 5, and a portion of each clone was grown further for chromosome counting and Southern (DNA) blot analysis.
23. The sex of strains PDFF1 to PDFF7 was determined by PCR, essentially as described [R. Griffiths and B. Tiwari, *Mol. Ecol.* **2**, 405 (1993); B. W. Kirkpatrick and R. L. Monson, *J. Reprod. Fert.* **98**, 335 (1993)].
24. Genomic DNA isolated from cloned pMIX1 transfectants was digested with Bam HI and Eco RI and subjected to Southern blot analysis with a 1.8-kb fragment of the BLG promoter.
25. PCNA analysis of serum-deprived cells was performed essentially as described [I. R. Kill *et al.*, *J. Cell Sci.* **100**, 869 (1991)].
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32. We thank J. Bracken and M. Malcolm-Smith for technical assistance in large animal work, H. Bowran and D. McGavin for care of the animals, D. Cottom for providing data on microinjection studies, the Animal Sciences Team at PPL Therapeutics for help in deriving the PDFF cells, Y. Gibson and the Small Animal Unit at PPL Therapeutics for generating FIX transgenic mice, S. Bruce for FIX protein analysis, G. G. Brownlee for human FIX genomic λ clones, D. Melton for PGKneo, A. R. Scott for technical assistance with molecular biology, and I. Garner for constructive discussions during the course of the project. The experiments were conducted under the Animals (Scientific Procedures) Act 1986 and with the approval of the Roslin Institute Animal Welfare and Experiments Committee.

12 September 1997; accepted 31 October 1997

into an FFF instrument (Model F-1000-FO, FFFractionation; LLC, Salt Lake City, UT) operating with 0.007% Triton X-100 in water mobile phase at 2 ml $min^{-1}$ and a cross-flow rate of 0.5 ml $min^{-1}$.

17. Electrodeposition was performed by placing 20 μl of the nanotube suspension on the surface of a freshly cleaved HOPG substrate (Advanced Ceramics, Cleveland, OH), confining the droplet within a viton O-ring (4-mm outer diameter, 1.7 mm thick), capping the trapped suspension with a stainless steel electrode on top of the O-ring, and applying a steady voltage of 1.1 V for 6 min. When suspended in water, the nanotubes are negatively charged and are therefore driven by the electric field onto the HOPG surface. After deposition, the HOPG-nanotube surface was washed with methanol on a spin coater to remove the water and Triton X-100 surfactant.
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20. We thank K. Smith and V. Colvin for helpful discussions. Supported by the NSF, the Office of Naval Research, the Advanced Technology Program of Texas, and the Robert A. Welch Foundation.

12 January 1998; accepted 27 March 1998

# Cloned Transgenic Calves Produced from Nonquiescent Fetal Fibroblasts

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An efficient system for genetic modification and large-scale cloning of cattle is of importance for agriculture, biotechnology, and human medicine. Here, actively dividing fetal fibroblasts were genetically modified with a marker gene, a clonal line was selected, and the cells were fused to enucleated mature oocytes. Out of 28 embryos transferred to 11 recipient cows, three healthy, identical, transgenic calves were generated. Furthermore, the life-span of near senescent fibroblasts could be extended by nuclear transfer, as indicated by population doublings in fibroblast lines derived from a 40-day-old fetal clone. With the ability to extend the life-span of these primary cultured cells, this system would be useful for inducing complex genetic modifications in cattle.

Research has been in progress for more than a decade to develop a system for genetic modification and large-scale cloning in cattle (*1*), an important species in agriculture, biotechnology, and human medicine. In the initial work on cloning, embryonic blastomeres were used as donor nuclei because they were thought to be relatively undifferentiated, readily reprogrammed, and likely to support full-term development of the fetus (*2*). Initial efforts at refining the methodology of nuclear transfer resulted in significant, but limited, improvements in efficiency, and at most, only a few identical calves could be produced from a single donor embryo because of the limited number of cells in the early embryo (*3*). The next step toward expanding the potential of cloning was the development and use of embryonic stem cells as a source of donor nuclei. Embryonic stem cells are derived from the inner cell mass of an early embryo and are thought to be relatively undifferentiated. In addition, mouse embryonic stem cells divide indefinitely in culture without differentiation and can be readily genetically modified (*4*). Embryonic stemlike cells have been developed in the bovine (*5*) and have been used as a source of donor nuclei in nuclear transfer, but they only supported development of fetuses to 60 days in vivo (*6*). To date, a source of cells that can be used for genetic modification and large-scale cloning in cattle has not been found.

Other research in nuclear transplantation has shown that the cell cycle stage of the donor cell affects the extent of development of the embryo after nuclear transfer. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated (*7*). This condensation phase has been shown to cause chromosomal defects in donor cells that are undergoing DNA synthesis (*7*). Donor cells in the $G_1$ phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development (*7*).

In previous work in the sheep, it was suggested that arrest in $G_0$ (by serum starvation) was the key in allowing donor somatic cells to support development of embryos to term (*8*).

Our rationale in selecting an optimal donor cell for nuclear transplantation was that the cell should not have ceased dividing (which is the case in $G_0$) but be actively dividing, as an indication of a relatively undifferentiated state and for compatibility with the rapid cell divisions that occur during early embryo development. The cells should also be in $G_1$, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long $G_1$ phase. We chose fibroblasts from fetuses because they can grow rapidly in culture and have an inherently long $G_1$ phase (*9*).

Fetal fibroblasts were isolated from a day 55 male fetus (Fig. 1A), cultured in vitro, and passaged twice before being transfected with a marker construct consisting of a β-galactosidase–neomycin resistance fusion gene driven by a cytomegalovirus (CMV) promoter (pCMV/β-GEO) (*10*). Cells were selected with neomycin for 2 weeks, and five neomycin-resistant colonies were isolated and analyzed for stable transfection by polymerase chain reaction (PCR) amplification of a segment of the transgene (*11*) and by assay of β-galactosidase activity. Colony CL1 was chosen for nuclear transfer experiments. These fibroblast cells

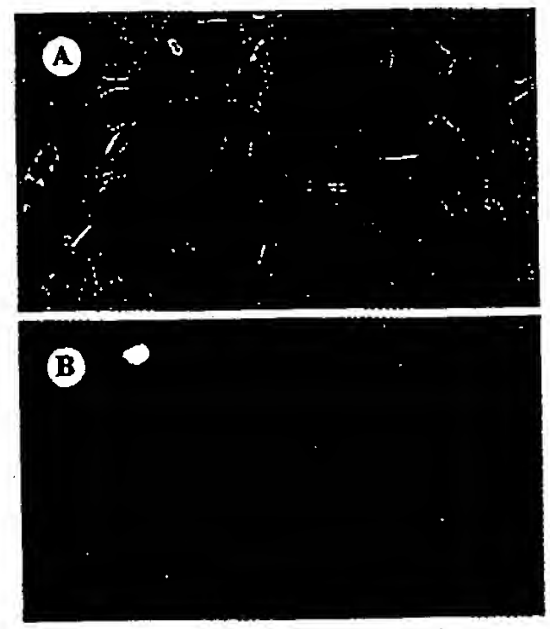


**Fig. 1.** Transgenic fetal fibroblast CL1-5 used for nuclear transplantation (**A**) phase contrast (×100). (**B**) Labeling of CL1-5 fibroblast cell line with PCNA monoclonal antibody (Sigma, St. Louis, MO) and FITC-conjugated secondary antibody (magnification × 200).

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were characterized as negative for cytokeratin, positive for vimentin (mesoderm origin), and negative for a human fibroblast cell surface marker.

A total of 276 nuclear transfer embryos were produced and 33 blastocysts (12%) were obtained after a week in culture (*12*). Twenty-eight of the blastocysts were nonsurgically transferred into 11 synchronized recipients (day 7 after onset of heat). Six cows were detected pregnant by ultrasound 40 days after nuclear transfer (55%), and five cows remained pregnant by day 60 of gestation (45%). No multiple pregnancies were produced. One cow aborted at day 249 of gestation. Its placenta was characterized as having hydroallantois, enlarged placentomes, and an edematous chorioallantois and amnion. Upon necropsy, the fetus was oversized (54 kg at month 8 of gestation), lung lobes were edematous, umbilical vessels were twice normal size, and the right heart ventricle was enlarged. The remaining four calves continued development to term. As controls, 122 in vitro–matured oocytes were parthenogenically activated, and after a week in culture 21 (18%) blastocysts were obtained.

Calves ACT2, ACT3, ACT4, and ACT5 were delivered at 277, 286, 287, and 289 days of gestation, respectively. Calf ACT2 died 5 days after birth as a result of pulmonary hypertension leading to insufficient pulmonary perfusion. Along with this pathology, the animal exhibited a dilated right ventricle, a patent ductus arteriosus, a pulmonary artery greater than the size of the aorta, and umbilical vessels three times normal size. The placenta also manifested abnormalities such as hydroallantois and a reduced number of enlarged placentomes. Calves ACT3 and ACT5 were delivered by cesarean section, and ACT4 was born vaginally. These three animals were phenotypically normal, and no abnormal placentation was revealed (Fig. 2). All five calves were screened by PCR amplification of a segment of the transgene (*11*), which confirmed that the cells were transgenic with the pCMV/βGEO gene (Fig. 3).

A restriction enzyme digest and Southern blot of genomic DNA (*13*) from calves ACT3, ACT4, and ACT5 demonstrated that these animals had an identical gene integration site and therefore were derived from the same fibroblast clone (Fig. 4). Also, the neomycin resistance gene was demonstrated to be functional in fibroblasts obtained from dermis of the calves. When cultured under selection with Geneticin, cells were able to survive for more than 10 days in culture (Fig. 5).

Developmental problems exhibited by the nonsurviving calves could be attributed to abnormal placentation. It remains to be determined whether the origin of this pathology can be connected to the nuclear transfer procedure itself or to the culture conditions in which the embryos were maintained during the first week of development. Previous data on cattle have indicated an association between in vitro culture conditions and calf abnormalities (*14*). In sheep, when nuclear transfer embryos were grown in vivo, perinatal loss occurred; however, no common factor could be attributed as to the cause of death (*8*).

Because it has been observed in rabbits that chemical synchronization can result in fewer successful pregnancies (*15*), CL1 bovine cells were not synchronized in $G_1$ but were constantly cultured with 10% fetal bovine serum and used at 70 to 80% confluence. Immunohistochemical analysis showed that 82% (279/340) of the cells were positive for proliferating cell nuclear antigen (PCNA) (Fig. 1B). Fluorescence-activated cell sorting (FACS) analysis revealed that, even though the cells were actively dividing, 56% of the cells were in $G_1$, providing a large population that could support development and precluding the need for an artificial synchronization procedure.

Our results indicate that an actively dividing population of cells can support development to term after nuclear transfer and that serum starvation is not a necessary treatment. Although our population of cells were actively dividing, we cannot determine which subpopulation ($G_1$, S, $G_2$, or M) may have produced the offspring. Certainly, more work will be necessary to fully understand the properties of somatic cells required to allow for successful reprogramming and full-term development of offspring.

The fibroblasts used in this study have a finite life-span in culture, which could limit the types of transgenic modifications that could be made. When cultured until senescence, fibroblasts derived from 6-week-old fetuses undergo 30 population doublings, with an average cell cycle length of 28 to 30 hours. As shown with the previous data, this number of population doublings is sufficient to generate clonally derived transgenic cell lines. However, many uses of genetically modified fetuses and animals will require gene targeting by homologous recombination. For homologous recombina-

**Fig. 2.** Normal cloned calves ACT3, ACT4, and ACT5, at 3 weeks of age.

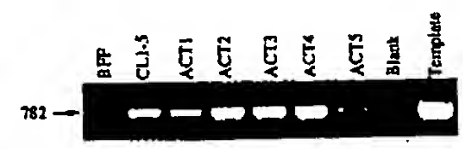


**Fig. 3.** Gel of PCR-amplified segment of the pCMV/βGEO construct (*15*) of DNA obtained from the original cell line and calf ear samples. BFF, nontransgenic fetal fibroblasts; CL1-5, original clonal cell line used for nuclear transfer; ACT1, fetus aborted at 249 days of gestation; ACT2, calf dead at 5 days after birth; ACT3, ACT4, and ACT5, normal calves. Size marker (in base pairs) is on the left.

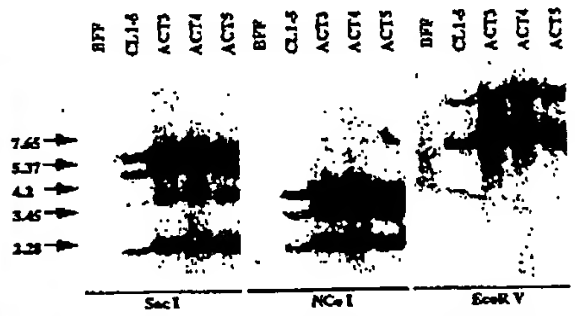


**Fig. 4.** Southern blot of genomic DNA (*16*) obtained from ear notches of live calves. DNA was cut with three different restriction enzymes: (**A**) Sac I, (**B**) Nco I, and (**C**) EcoR V. Size markers (in kilobase pairs) are on the left.

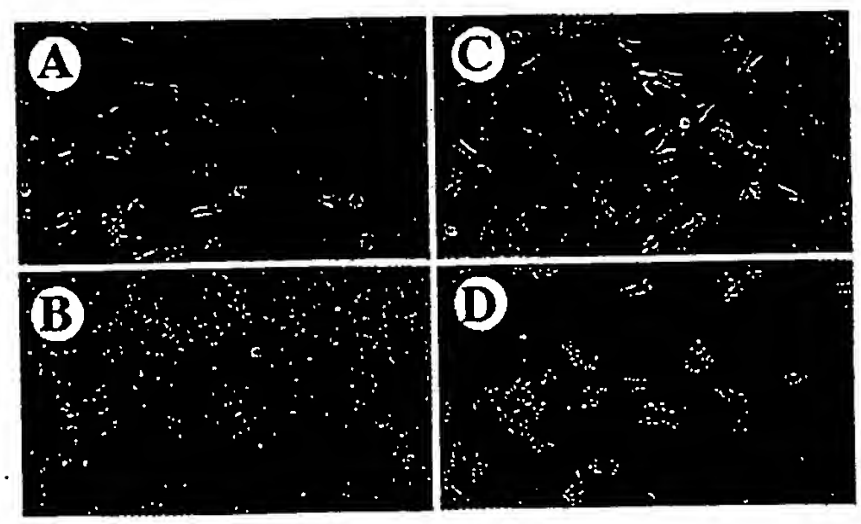


**Fig. 5.** Fibroblast from ACT4 calf and nontransgenic fetal fibroblast cultured with Geneticin (300 μg/ml, Sigma). (**A**) ACT4 calf fibroblasts isolated from dermis at day 1 and (**B**) day 10. (**C**) Nontransgenic fetal fibroblast at day 1 and (**D**) day 10.

tion, transgenic cells are first selected, but then a second round of selection is necessary to identify the correctly targeted cells. The two rounds of selection will require a greater number of cell divisions, and the cells could easily become senescent by the time the correctly targeted cells are identified, and they would certainly be senescent before a homozygous mutant is produced. To address this problem, we generated a 40-day-old fetus using the CL1 cell line at 0.8 population doubling from senescence. The fetus was removed from the uterus, and fibroblasts were derived from it. The number of population doublings until senescence was 31 and 33 for the nuclear transfer and same-age nonmanipulated fetal fibroblasts, respectively. These data suggest that fibroblast life-span can be enhanced by nuclear transfer. This approach could enable us to generate as many gene targeting events as needed by subjecting the cell line to the successive rounds of nuclear transfer.

This somatic cell nuclear transfer procedure could improve the efficiency of producing transgenic cattle and broaden the scope of applications for transgenic cattle. With previous microinjection techniques, about 500 embryos would have to be injected and transferred to recipient cows to get one transgenic offspring (16). For the nuclear transfer technique with transgenic somatic cells, the transfer of nine embryos to four cows produced a transgenic offspring, greatly reducing the time and costs involved. With the nuclear transfer approach, an entire herd of the appropriate sex transgenic cattle could be produced in one generation, whereas the traditional microinjection approach would require at least two generations, and likely more, to obtain a production herd. This is a savings of 2 years for each generation. Finally, the somatic cell nuclear transfer approach could broaden the scope of use of transgenic cattle because it allows the targeting of DNA inserts to specific sites in the genome. This is important for deleting or replacing bovine genes that might interfere with human protein isolation or cause rejection of grafted tissues. Inserting genes into a selected site could be used to ensure tissue-specific and consistent expression levels of transgenes. Furthermore, insertion of genes into the same site in multiple lines of animals could be used to quickly generate homozygous lines of animals while avoiding inbreeding.

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9. D. M. Gadbois *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8626 (1992).
10. The plasmid pCMV/βGEO was constructed by inserting the βGEO fusion fragment [Xho I to Xba I [G. Fiedrich *et al.*, *Genes Dev.* **5** (no. 9), 1513 (1991)]] into pcmvβ (Clonotech) between Not I sites.
11. The transgene was detected in transfected cells, fetuses, and tissue from adult animals by PCR with a 21-base sense primer (ACT3βGEO) 5′-CGCTGTGGTACACGCTGTGCG-3′, and a 22-base antisense primer (ACT4βGEO) 5′-CACCATCCAGTGCAGGAGCTCG-3′ (Amitof Biotech). Reactions were run for 35 cycles, with denaturation at 95°C for 30 s, annealing at 65°C for 1 min, followed by extension for 2 min at 72°C. A final extension for 10 min at 72°C was included after the last cycle. The amplified product was a 782–base pair (bp) fragment. The samples were analyzed by separating them by size in a (1%) tris-borate EDTA agarose gel containing ethidium bromide.
12. For nuclear transfer, bovine oocytes were aspirated from slaughter-collected ovaries, put in maturation media [P. Damiani *et al.*, *Mol. Reprod. Devel.* **45**, 521 (1996)], and shipped to the laboratory overnight at 38.5°C. Oocytes were mechanically enucleated at 18 hours after maturation, and chromosome removal was assessed with bisBENZIMIDE (Hoechst 33342; Sigma, St. Louis, MO) dye under ultraviolet light. Successfully enucleated oocytes were fused with actively dividing CL1 fibroblasts by using one electrical pulse of 180 volts/cm for 15 μs (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 2 to 4 hours, oocytes were then chemically activated with calcium ionophore (5 μM) for 4 min (catalog number 407952; Cal Biochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 with bovine serum albumin (BSA) (3 mg/ml) for 3 hours (fatty acid free, Sigma) [J. L. Susko-Parrish *et al.*, *Devel. Biol.* **166**, 729 (1994)]. After activation, eggs were washed in hamster embryo culture medium (HECM)–Hepes five times and placed, for culture, in a 500-μl well of CR2 (Specialty Media, Lavallette, NJ) with BSA (3 mg/ml) (fatty acid free) along with $1 \times 10^6$ mouse embryonic fibroblasts (MEFs) per milliliter. Incubation was performed for 6.5 days at 38.5°C and 5% $CO_2$ in air, and selected embryos were shipped overnight in a portable incubator to the embryo transfer facility.
13. Calf tissue samples (peripheral ear tissue) were prepared for Southern blot analysis according to P. Laird *et al.* [*Nucleic Acids Res.* **19**, 4293 (1991)]. Ten micrograms of genomic DNA was run in a 0.75% agarose gel in tris-acetate buffer, transferred onto Zetabind (Cuno, Meriden, CT), cross-linked by ultraviolet light, prehybridized for 4 hours, then hybridized at 42°C for 8 hours. The blot was washed and exposed to Biomax film (Kodak). A probe of 3881 bp, EcoR I–EcoR I DNA fragment of βGEO was labeled with $^{32}$P-deoxycytidine triphosphate (dCTP) by using a random primed labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).
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17. We thank J. Balise, R. Duby, J. Morris, N. Kieser, E. Dickinson, and N. Ward Mackall for their technical assistance; G. Morgan for cell cycle studies; J. Hill, S. R. Bumgardner, and A. J. Russel for attending calves at birth; and C. Looney for embryo transfer work. Supported by a grant from Advanced Cell Technology to the University of Massachusetts.

18 February 1998; accepted 7 April 1998


# A Signaling Complex of $Ca^{2+}$-Calmodulin–Dependent Protein Kinase IV and Protein Phosphatase 2A


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Stimulation of T lymphocytes results in a rapid increase in intracellular calcium concentration ($[Ca^{2+}]_i$) that parallels the activation of $Ca^{2+}$-calmodulin–dependent protein kinase IV (CaMKIV), a nuclear enzyme that can phosphorylate and activate the cyclic adenosine monophosphate (cAMP) response element–binding protein (CREB). However, inactivation of CaMKIV occurs despite the sustained increase in $[Ca^{2+}]_i$ that is required for T cell activation. A stable and stoichiometric complex of CaMKIV with protein serine-threonine phosphatase 2A (PP2A) was identified in which PP2A dephosphorylates CaMKIV and functions as a negative regulator of CaMKIV signaling. In Jurkat T cells, inhibition of PP2A activity by small t antigen enhanced activation of CREB-mediated transcription by CaMKIV. These findings reveal an intracellular signaling mechanism whereby a protein serine-threonine kinase (CaMKIV) is regulated by a tightly associated protein serine-threonine phosphatase (PP2A).


Cellular responses to external signals require coordinated control of protein kinases and phosphatases; multiple complexes containing both intracellular signaling enzymes are likely to be important for the regulation and specificity of signal transduction pathways. The targeting of protein kinases and phosphatases to specific subcellular compartments, through association with scaffold proteins such as A-kinase anchoring proteins, may contribute to the specificity of cellular signaling (*1*). However, the enzymes are retained by the anchoring protein in their inactive state (*2*), and potential regulatory interactions within these multiprotein complexes remain unknown. Preexisting com-

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# Sequential targeting of the genes encoding immunoglobulin-μ and prion protein in cattle

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**Gene targeting is accomplished using embryonic stem cells in the mouse but has been successful, only using primary somatic cells followed by embryonic cloning, in other species. Gene targeting in somatic cells versus embryonic stem cells is a challenge; consequently, there are few reported successes and none include the targeting of transcriptionally silent genes or double targeting to produce homozygotes. Here, we report a sequential gene targeting system for primary fibroblast cells that we used to knock out both alleles of a silent gene, the bovine gene encoding immunoglobulin-μ (*IGHM*), and produce both heterozygous and homozygous knockout calves. We also carried out sequential knockout targeting of both alleles of a gene that is active in fibroblasts, encoding the bovine prion protein (*PRNP*), in the same genetic line to produce doubly homozygous knockout fetuses. The sequential gene targeting system we used alleviates the need for germline transmission for complex genetic modifications and should be broadly applicable to gene functional analysis and to biomedical and agricultural applications.**

Gene targeting by homologous recombination is a powerful method of specifically modifying a gene of interest used extensively for gene functional analysis in mice[1–3]. Gene targeting is accomplished in the mouse using embryonic stem (ES) cells, but in essentially all other species, ES cells suitable for gene targeting are not available. The few reports on gene targeting in other mammalian species used primary somatic cells followed by embryonic cloning[4–7]; in some instances, the embryos were then used to produce cloned offspring. Gene targeting in primary somatic cells is a challenge[8–12] because somatic cells have a relatively short lifespan, which limits selection of properly targeted cell colonies, and a low frequency of homologous recombination[11] compared with mouse ES cells. Because of these limitations, success in somatic cell gene targeting has been achieved for only a couple of genes that were transcriptionally active in the cell line used for targeting and only in sheep and pig. Transcriptionally active genes are more amenable to gene targeting than silent genes, because they have a higher frequency of homologous recombination[5,8] and correctly targeted cells can be easily selected by having the targeted gene promoter drive expression of a selection marker. Application of this 'promoter-less' positive selection[4–7] is limited to transcriptionally active genes in the somatic cells.

To fully evaluate the consequences of a genetic modification, both alleles of the gene must be targeted. In mice, this is generally done by breeding heterozygous knockout founders to produce a homozygous knockout inbred line. But breeding to homozygosity is severely impeded in species that have a long generation interval, such as cows, sheep and pigs, and that are negatively impacted by the consequences of inbreeding. In pigs, two innovative approaches have been used to circumvent the long generation interval and low rate of homologous recombination for targeting the second allele of the gene encoding α-(1,3)-galactocyltransferase. Heterozygous knockout fibroblasts were selected *in vitro* for lacking enzymatic activity resulting either from a spontaneous point mutation in the second allele of the gene[13] or from mitotic recombinants[14]. Unfortunately, these approaches are neither useful for silent genes nor widely applicable for active genes.

In this study, we developed a broadly applicable and rapid method for generating multiple gene targeting events in cattle. The method consists of sequential application of gene targeting by homologous recombination and rejuvenation of cell lines by production of cloned fetuses (**Fig. 1**). We used this procedure to demonstrate the first successful targeting of a transcriptionally silent gene and production of both heterozygous and homozygous knockout calves. We also targeted a second gene, resulting in doubly homozygous knockout bovine fetuses and cell lines.

## RESULTS

### Targeting the first allele of *IGHM*

We chose to target *IGHM*, which is transcriptionally silent in fibroblasts. We characterized this gene in a male Holstein fetal fibroblast cell line (6939) to identify a polymorphic marker DNA sequence, outside the knockout vector sequence, that could be used to distinguish the two alleles (allele A and allele B; **Fig. 2a**). We constructed the first knockout vector using *IGHM* genomic fragments from around the

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Published online 6 June 2004; doi:10.1038/ng1373

constant μ exon 2 region, which was derived from a nonisogenic Holstein genomic library. The knockout vector used to target the first allele contained a diphtheria toxin A (DT-A) gene[15] as a negative selection marker and a *puro* selection marker driven by a mouse PGK promoter, flanked by *lox*P sequences and followed by a transcriptional and translational STOP[16] cassette (pBCμΔKOpuro; Fig. 2a). We electroporated fetal fibroblasts from cell line 6939 with the first knockout vector to produce 446 wells resistant to puromycin. We split the wells on day 14 and screened half the cells by PCR (primer pairs puroF2 × puroR2; Fig. 2a) to identify wells containing correctly targeted cells. Initially, six wells seemed to contain correctly targeted cells. To exclude wells giving a false positive result, we subjected all the PCR products to bidirectional sequencing analysis with the puroF2 and puroR2 primers. Two wells (147 and 384; 0.45%) were correctly targeted and contained heterozygous *IGHM* knockout ($IGHM^{+/-}$) cells. On the basis of polymorphic differences identified by sequence analysis, we determined that the knockout vector was integrated into allele A in well 384 and into allele B in well 147.

### Generating $IGHM^{+/-}$ fetuses and calves

We used the remaining cells from the two wells for embryonic cloning to generate fetuses and rejuvenate the cell lines. Pregnancy rate at 40 days of gestation was 50% (15 of 30, two embryos per recipient; Table 1), and at 60 days of gestation, we collected six fetuses and reestablished fibroblasts. Three of six fetuses (2184-1, 2184-2 and 3287) were $IGHM^{+/-}$ (Fig. 2b) as confirmed by the PCR (primer pairs puroF2 × puroR2) and sequence analysis. Nontargeted fetuses probably resulted either from nontargeted cells that coexisted with the targeted cells in the wells or from loss of the transgene due to lack of selection pressure during fetal development. Both fetuses 2184-1 and 2184-2 were derived from well 384, where the knockout vector was integrated into allele A, and fetus 3287 was from well 147, where the knockout vector was integrated into allele B. We produced cloned $IGHM^{+/-}$ embryos from all three regenerated cell lines and transferred them to 153 recipients to produce 13 (8%, Table 1) healthy $IGHM^{+/-}$ calves, whose genotypes were confirmed by PCR (Fig. 2c) and sequence analysis (data not shown).

### Targeting the second allele of *IGHM*

To target the second allele of *IGHM*, we prepared a second knockout vector in which the *puro* selection marker was replaced with a *neo* gene driven by an ST (SV40 promoter and thymidine kinase enhancer) promoter. In attempting to target the second allele of a gene, there is the possibility that the targeting vector will undergo homologous recombination with the integrated targeting vector, resulting in replacement of the knockout vector in the previously targeted allele rather than disruption of the intact allele. This is a problem particularly if the first targeting vector has a strong bias for one allele. This was not observed with our first, nonisogenic, knockout vector, indicating either that the two alleles had similar sequences or that polymorphisms had an equal effect on targeting efficiency. We assumed the latter and determined whether the frequency of targeting of allele A could be enhanced by constructing a second knockout vector in which the short homologous arm was replaced with a PCR-derived sequence amplified directly from allele A of the cell line 6939 (this vector was designated pBCμΔNKOneo).

We used all three $IGHM^{+/-}$ cell lines (2184-1 and 2184-2, targeted in allele A; 3287, targeted in allele B) for targeting with the second knockout vector (Fig. 2a). In cell lines 2184-1 and 2184-2, we screened 1,211 wells resistant to G418 by PCR (primer pairs neoF3 × neoR3; Fig. 2a) and then carried out sequence analysis. Five wells contained correctly targeted cells. In two of them (0.17%), the vector was integrated into the intact allele B, producing homozygous knockout ($IGHM^{-/-}$) cells, and in three wells, the targeting vector in allele A was replaced. In cell line 3287, we screened 569 wells resistant to G418 by PCR (primer pairs neoF3 × neoR3; Fig. 2a) and then carried out sequence analysis. Seven wells contained correctly targeted cells. In six of them (1.1%), the vector was integrated into the intact allele A, producing $IGHM^{-/-}$ cells, and in one well, the targeting vector in allele B was replaced. Overall, the vector had a bias of 6:1 for intact allele A to allele B and was more efficient for homozygous targeting when used with cell line 3287 in which allele B was first targeted, as expected.

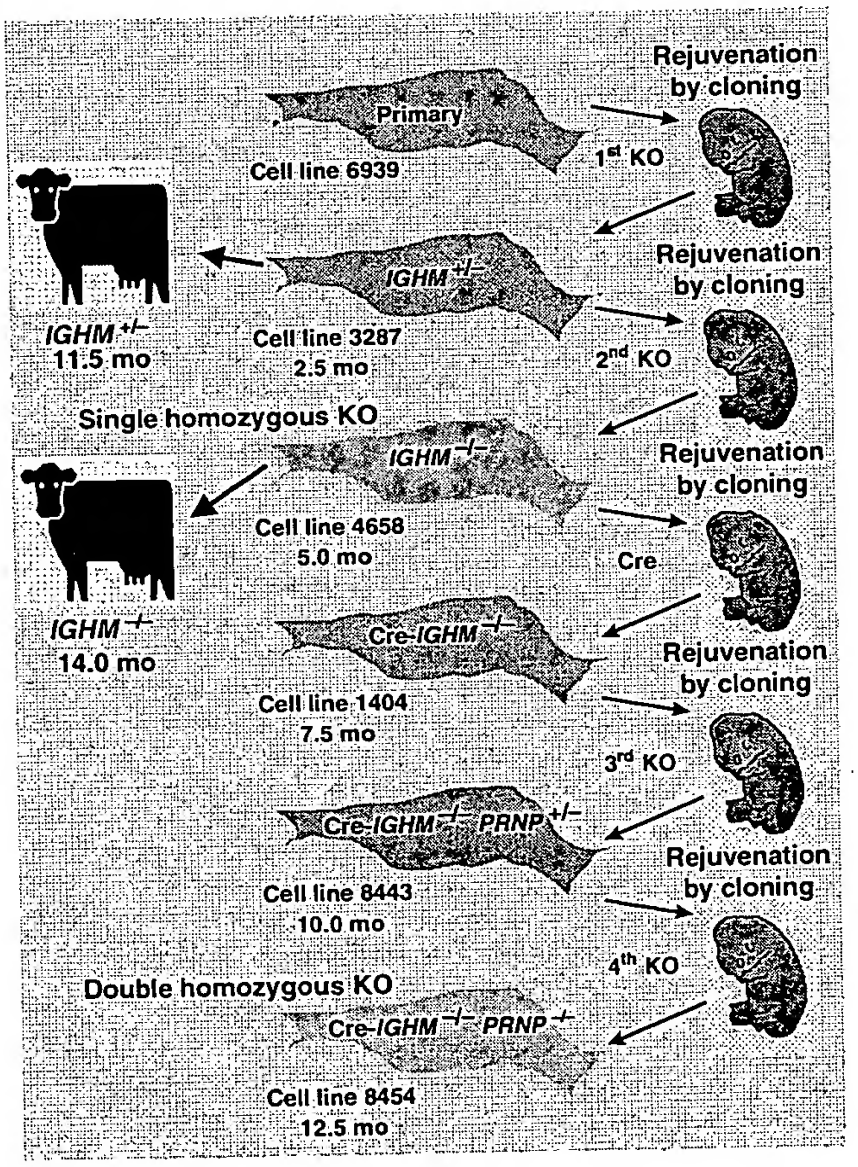


**Figure 1** Procedure for sequential gene targeting in bovine primary fibroblasts. Holstein fetal fibroblasts (6939) were targeted and wells containing targeted cells were then selected and cloned to generate $IGHM^{+/-}$ fetuses. The $IGHM^{+/-}$ cell line (3287) was then used to produce calves and to target the second allele of *IGHM*. Once again, cells were selected and regenerated by production of fetuses. Fetuses were collected to produce $IGHM^{-/-}$ cell lines, analyze *IGHM* expression and produce calves. An $IGHM^{-/-}$ cell line (4658) was transfected with a Cre-recombinase expression plasmid to remove both *neo* and *puro* genes simultaneously. A third round of embryonic cloning then generated cloned fetuses and cell lines in which both *neo* and *puro* selection marker genes were excised. One Cre-excised $IGHM^{-/-}$ fibroblast cell line (1404) was used for a third round of gene targeting to produce triply targeted Cre-$IGHM^{-/-}$ $PRNP^{+/-}$ fetuses and cell lines. One cell line (8334) was subjected to the fourth round of gene targeting to produce doubly homozygous knockout (Cre-$IGHM^{-/-}$ $PRNP^{-/-}$) fetuses and cell lines and to analysis of *PRNP* expression. A representative time line for each step is indicated. KO, knockout.

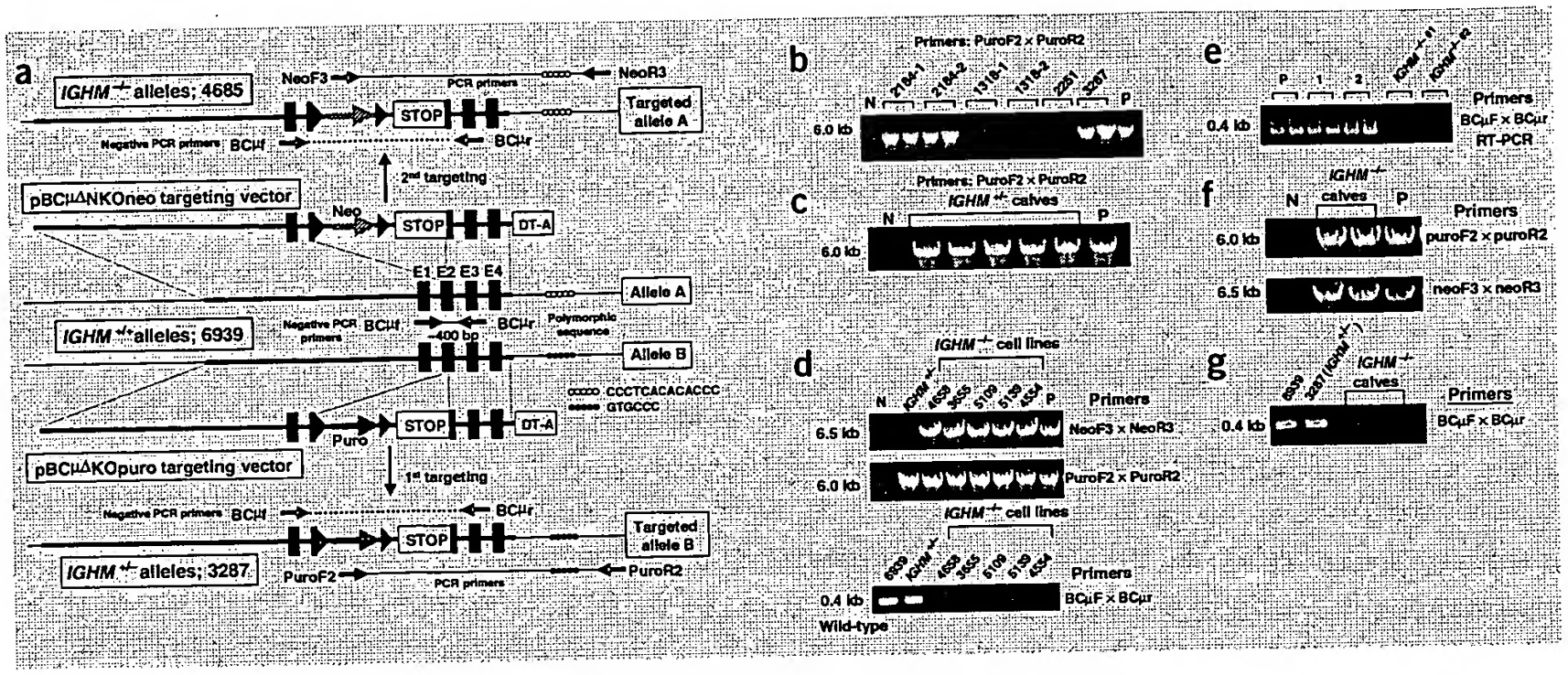


**Figure 2** Sequential targeting of *IGHM* in primary bovine fibroblasts. (**a**) Structure of *IGHM* constant region locus in cell line 6939, the *puro* and *neo* vectors used for the first and second round of targeting, respectively, and the genomic PCR assay used for the first and second targeting events. In fibroblasts of cell line 6939, polymorphic sequences were found to distinguish allele A and allele B, as indicated. (**b**) Identification of $IGHM^{+/-}$ fetuses by genomic PCR. N, negative control; P, positive control. Cell lines 2184-1, 2184-2 and 3287 were $IGHM^{+/-}$. (**c**) Genotyping of $IGHM^{+/-}$ calves by genomic PCR. N, negative control; P, positive control. Five $IGHM^{+/-}$ calves were genotyped and all contained correctly targeted cells from the first targeting event. (**d**) Identification of $IGHM^{-/-}$ fetuses and fibroblasts by genomic PCR. N, negative control; P, positive control. 6939 is the original fibroblast cell line. Cell lines 4658, 3655, 5109, 5139 and 4554 contained correctly targeted cells from both targeting events but no wild-type alleles. (**e**) RT-PCR analysis of *IGHM* expression in mRNA extracted from spleen in 90-d-old fetuses. Clear expression was detected from a positive control (P) and the wild-type (6939) fetuses but not from $IGHM^{-/-}$ fetuses. (**f**,**g**) Genotyping of $IGHM^{-/-}$ calves by genomic PCR. N, negative control; P, positive control. (**f**) Two $IGHM^{-/-}$ calves were genotyped and contained correctly targeted cells from targeting events at both alleles but (**g**) no wild-type alleles.

### Generating $IGHM^{-/-}$ fetuses and calves

We selected two $IGHM^{-/-}$ wells (76 and 91) derived from cell line 3287 for embryonic cloning to generate fetuses and rejuvenate the cell lines. Overall pregnancy rate for $IGHM^{-/-}$ fetuses at 40–50 days of gestation was 45% (40 of 89; **Table 1**). At 45 days of gestation, we collected and evaluated 5 fetuses derived from well 76 and 15 fetuses from well 91. All 5 from well 76 (**Fig. 2d**) and 3 of 15 from well 91 (data not shown) contained correctly targeted cells specific for the first and second targeting events (primer pairs puroF2 × puroR2 and neoF3 × neoR3), as shown by PCR. PCR results were confirmed by sequence analyses and negative PCR[17] results (primer pairs bCμf × bCμr; **Fig. 2a**) for the wild-type alleles (**Fig. 2d**). We confirmed functional knockout by generating 90-day fetuses from regenerated $IGHM^{-/-}$ fibroblasts and evaluating *IGHM* expression in spleen cells. Absence of expression was confirmed by RT-PCR (primers pairs bCμf × bCμr; **Fig. 2e**). We created cloned embryos from five $IGHM^{-/-}$ cell lines and transferred them to recipients for development to term. Eight calves (6%; **Table 1**) were born recently and were confirmed to be $IGHM^{-/-}$ by PCR (**Fig. 2f**) and sequence analyses (data not shown), verifying that sequential gene targeting and successive rounds of cell rejuvenation are compatible with full-term development of healthy homozygous knockout calves (**Fig. 2g**).

**Table 1 Production of cloned fetuses and calves from *IGHM*- and *PRNP*-targeted fibroblasts**

| Type of modification | End point[a] | Number of recipients implanted | Number of pregnancies at 40–45 d (%) | Number of live calves (%) |
|---|---|---|---|---|
| $IGHM^{+/-}$ | Fetus | 30 | 15 (50) | – |
| $IGHM^{+/-}$ | Calf | 153 | 99 (65) | 13 (8) |
| $IGHM^{-/-}$ | Fetus | 89 | 40 (45) | – |
| $IGHM^{-/-}$ | Calf | 137 | 86 (63) | 8 (6) |
| Cre/$IGHM^{-/-}$ | Fetus | 60 | 21 (35) | – |
| Cre/$IGHM^{-/-}$/$PRNP^{+/-}$ | Fetus | 39 | 28 (71) | – |
| Cre/$IGHM^{-/-}$/$PRNP^{-/-}$ | Fetus | 67 | 46[b] (68) | – |

[a]Fetuses were produced from selected colonies and calves were produced from rejuvenated cryopreserved cell lines. [b]After removing fetuses from 26 pregnant recipients, 15 pregnancies were left to continue to full term and 9 of them were confirmed pregnant at 60 d.

### Excising *neo* and *puro* in $IGHM^{-/-}$ fibroblasts

Sequential gene targeting requires a strategy for antibiotic selection of a newly integrated targeting vector in a cell line that already contains one or multiple antibiotic selection markers. The simplest approach is to use a different selection marker gene for each targeting event, but this approach limits the number of targeting events that may take place in a cell line. Another approach is to remove the selection markers using a Cre-*lox*P recombination system, as has been done in mouse ES cells[18]. Unexpectedly, the selection marker genes were not expressed in our regenerated *IGHM*-targeted fibroblasts, probably because reprogramming of the fibroblasts after embryonic cloning silenced the newly integrated sequence as part of the silent *IGHM* locus. Although selection marker removal was not necessary for further targeting in our $IGHM^{-/-}$ fibroblasts, we evaluated whether it was possible to remove the selection markers by transfection with a Cre recombinase expression plasmid. Because we intended

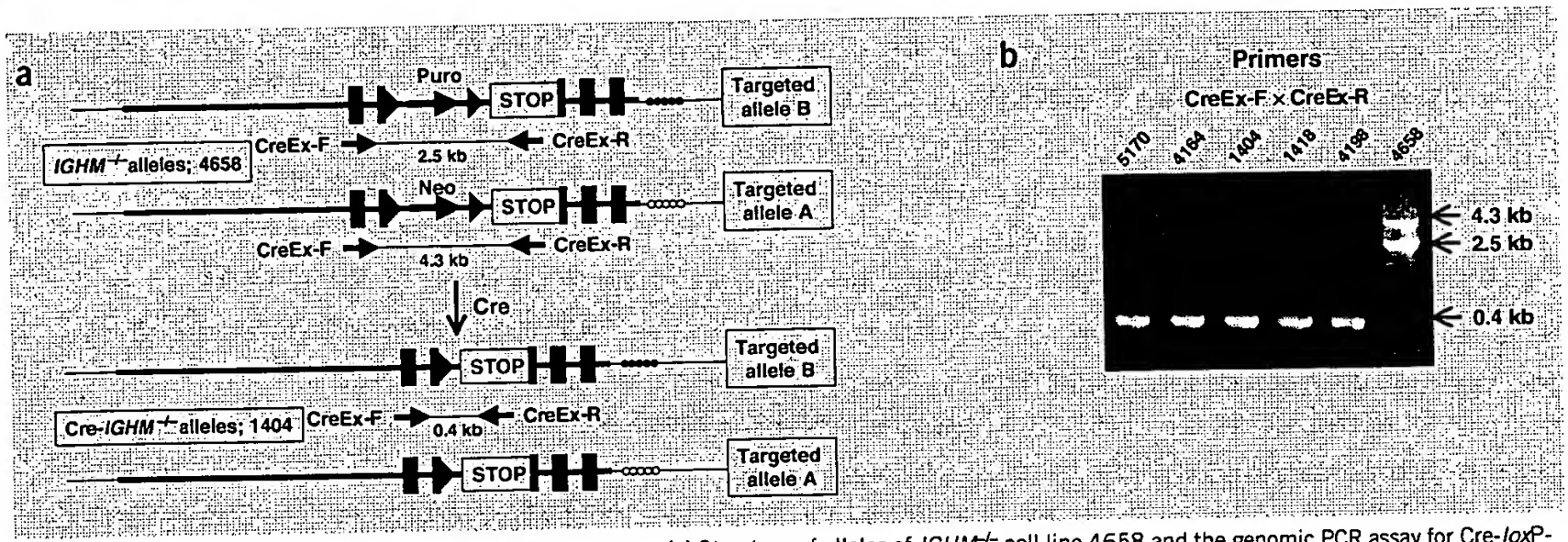


**Figure 3** Removal of both *neo* and *puro* genes by Cre-*lox*P system. (**a**) Structure of alleles of *IGHM*$^{-/-}$ cell line 4658 and the genomic PCR assay for Cre-*lox*P-mediated removal of selection marker genes. (**b**) Identification of Cre-*IGHM*$^{-/-}$ fetuses and fibroblasts by genomic PCR. Before introduction of Cre, 2.5-kb (*puro*) and 4.3-kb (*neo*) PCR products were detected in cell line 4658. A 0.4-kb band is detected in five Cre-excised fetuses.

Cre recombinase to be expressed transiently, we used a circular plasmid and restricted antibiotic selection to the first 3 days of culture. We used bovine *IGHM*$^{-/-}$ cell line 4658 for transfection and evaluated 24 selected wells by PCR for excision of the antibiotic selection genes from the targeted alleles (Fig. 3a). Multiple wells showed evidence of excision of both *puro* and *neo* genes, and we chose one for fetal cloning and regeneration of cell lines. Pregnancy rate at 40–50 days of gestation was 35% (21 of 60; Table 1). We recovered five fetuses, all of which had both selection markers removed (Fig. 3b), but all except fetus 1404 had the Cre recombinase plasmid integrated into the genome (data not shown). These results indicate that Cre-*lox*P recombination can be used to remove selection markers in somatic cells. Routine use in this system, however, will require improvements to reduce the integration frequency of the Cre expression plasmid.

## Targeting the first allele of *PRNP*

To evaluate the possibility of sequentially targeting a second gene, we subjected Cre-excised *IGHM*$^{-/-}$ (Cre-*IGHM*$^{-/-}$) fibroblasts (cell line 1404) to a third round of targeting to disrupt *PRNP*. We first characterized this gene to identify a polymorphic sequence, outside the knockout vector sequence, to distinguish the two alleles (allele C and allele D; Fig. 4a). The vector comprised nonisogenic sequences derived from the region around exon 3 of *PRNP* and the DT-A gene, the *neo* selection marker driven by the ST promoter, flanked by *lox*P sequences and followed by the STOP cassette (pBPrP(H)KOneo; Fig. 4a). We transfected cells with the third knockout vector and screened 203 G418-resistant wells by PCR. We identified 13 (6.4%) wells with cells that had a heterozygous knockout in *PRNP* on the Cre-*IGHM*$^{-/-}$ background (Cre-*IGHM*$^{-/-}$ *PRNP*$^{+/-}$; primer pairs neoF7 × neoR7; Fig. 4a

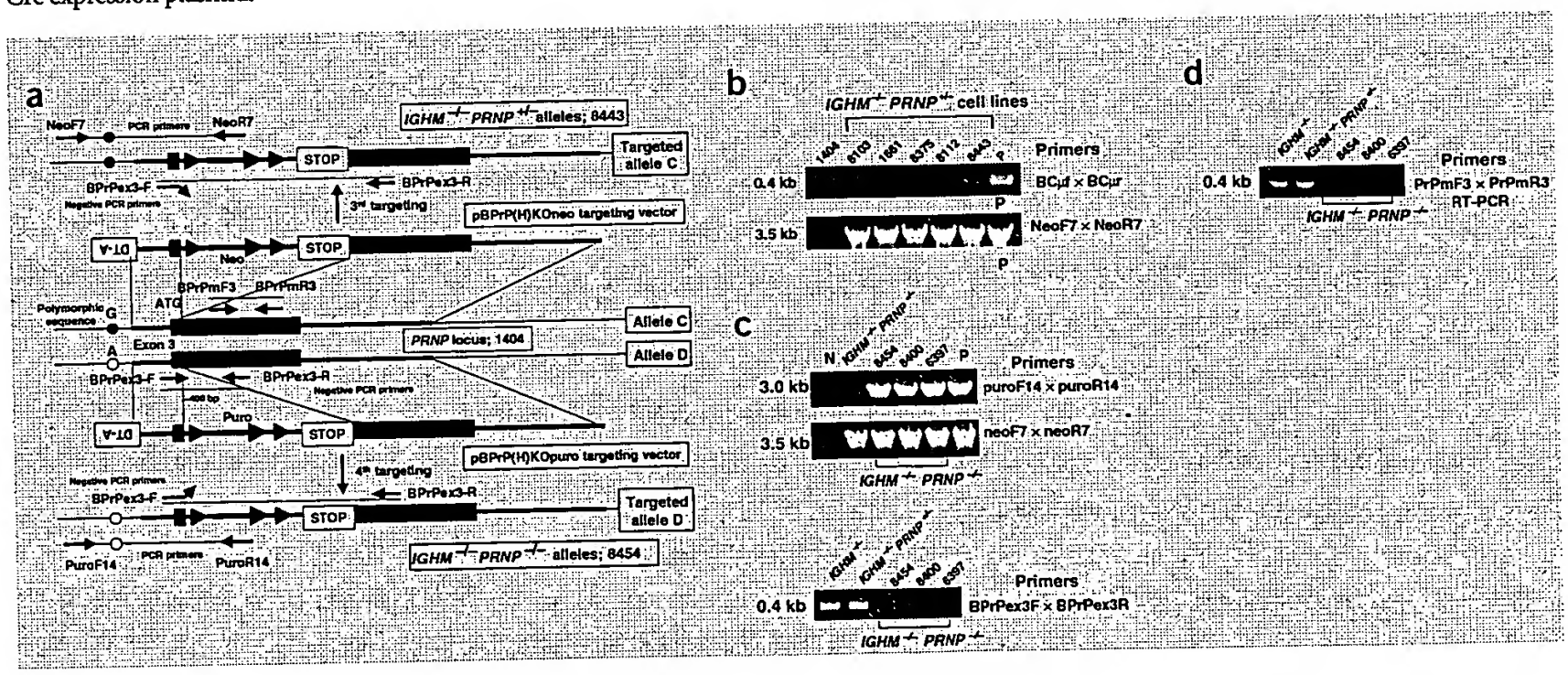


**Figure 4** Sequential targeting of *PRNP* in *IGHM*$^{-/-}$ fibroblasts. (**a**) Structure of the *PRNP* locus in Cre-*IGHM*$^{-/-}$ cell line 1404, *neo* and *puro* vectors used for the third and fourth rounds of targeting and the genomic PCR assay. (**b**) Identification of the triply targeted fetuses and fibroblast cell lines by positive and negative genomic PCR. P, positive control; cell line 1404, negative control. Cell lines derived from fetuses 8103, 1661, 8375, 8112 and 8443 contained correctly targeted cells from *PRNP* targeting and no wild-type *IGHM* alleles. (**c**) Identification of doubly homozygous knockout fibroblasts by genomic PCR. P, positive control; N, negative control. *IGHM*$^{-/-}$ fetal cell line 4658 and Cre-*IGHM*$^{-/-}$ *PRNP*$^{+/-}$ cell line 8443 are indicated. Cell lines 8454, 8400 and 6397 contained correctly targeted cells from the third and fourth targeting events but no wild-type alleles. (**d**) RT-PCR analysis of doubly homozygous knockout fetuses. Expression was observed in fetuses 4658 and 8443 but not in doubly homozygous knockout fetuses.

and data not shown). Sequence analysis showed that the third knockout vector was integrated into allele C of *PRNP* in all the positive wells. We used some wells for cloning to generate 28 pregnancies at 45 days of gestation (71%; **Table 1**). We collected five fetuses, all of which contained correctly targeted cells with the vector integrated into allele C of *PRNP*, as confirmed by PCR (primer pairs neoF7 × neoR7; **Fig. 4b**) and sequencing analyses (data not shown). Furthermore, we detected no amplification of wild-type *IGHM* alleles (primer pairs bCμf × bCμr; **Fig. 4b**), as expected. Targeting efficiency for *PRNP*, which is transcriptionally active in bovine fibroblasts, was substantially higher than for *IGHM* (6.4% versus 0.63%, respectively), which is not expressed in fibroblast cells.

### Targeting the second allele of *PRNP*

To examine the feasibility of quadruple targeting to produce doubly homozygous knockout fetuses and cell lines, we transfected the triply targeted cell line (8443, Cre-*IGHM*$^{-/-}$ *PRNP*$^{+/-}$) with a fourth knockout vector for the remaining allele of *PRNP*. We constructed the vector by replacing the *neo* gene with the *puro* gene (pBPrP(H)KOpuro; **Fig. 4a**) in the *PRNP* targeting vector used for the first allele. After selection and PCR screening (primer pairs puroF14 × puroR14; **Fig. 4a**), 17 (5.2%) wells contained targeted cells. Sequence analysis confirmed that the fourth knockout vector was integrated into allele D of *PRNP*, creating doubly homozygous knockout (Cre-*IGHM*$^{-/-}$ *PRNP*$^{-/-}$) cells, in 16 wells. In the remaining well, the targeted sequence in allele C was replaced. We used cells from correctly targeted Cre-*IGHM*$^{-/-}$ *PRNP*$^{-/-}$ wells for cloning to produce fetuses. The pregnancy rate derived from these embryos at 45 days of gestation was 68% (**Table 1**). We collected 18 fetuses, which were Cre-*IGHM*$^{-/-}$ *PRNP*$^{-/-}$, as confirmed by PCR analysis using the targeting event-specific primer pairs puroF14 × puroR14 and neoF7 × neoR7 (**Fig. 4c**). Sequencing analyses confirmed integration of the third (*neo*) and fourth (*puro*) *PRNP* targeting vectors into alleles C and D, respectively. Furthermore, we carried out a negative PCR analysis to confirm the absence of wild-type *PRNP* alleles (primer pairs BPrPex3F × BPrPex3R; **Fig. 4c**) and *IGHM* alleles (primer pairs bCμf × bCμr; data not shown); as expected, all four knockouts were confirmed. To evaluate *PRNP* mRNA expression, we examined fibroblasts from one *IGHM*$^{-/-}$ fetus, one Cre-*IGHM*$^{-/-}$ *PRNP*$^{+/-}$ fetus and three Cre-*IGHM*$^{-/-}$ *PRNP*$^{-/-}$ fetuses by RT-PCR. Functional disruption of *PRNP* expression was confirmed (**Fig. 4d**). These results indicate that multiple rounds of gene targeting, both for transcriptionally active and silent genes, were readily accomplished in a single somatic cell line using a cell rejuvenation approach.

## DISCUSSION

In this study we demonstrate, for the first time, a sequential gene targeting strategy for primary somatic cells, which can be used for targeting multiple alleles of a gene or for targeting multiple genes. The system proved effective for targeting both transcriptionally silent and active genes, demonstrating broad application, and was compatible with development of healthy calves through at least two rounds of gene targeting. There was no indication that additional rounds of gene targeting compromised development of cloned embryos, as judged from pregnancy rates at 45–60 days of gestation (**Table 1**). Pregnancies with the doubly homozygous knockout fetuses are in progress and pregnancy rates are consistent with the results obtained in this study.

One advantage of the sequential gene targeting system is that the time required to produce an animal with multiple genetic modifications is greatly reduced compared with traditional breeding strategies. With sequential gene targeting, each targeting event required ~2.5 months from transfection to establishment of regenerated cell lines; therefore, homozygous targeted calves could be created in 14 months (5 months for targeting two alleles and 9 months of gestation) and doubly homozygous targeted calves, including Cre-mediated excision of selection genes, could be created in 21.5 months (**Fig. 1**). In contrast, for cattle, breeding a heterozygous founder to produce homozygous calves would require ~5 years and generation of double homozygotes from two heterozygous founders is impractical.

Several factors were important for maximizing targeting efficiency and for successfully producing rejuvenated cell lines and calves. Overall, frequency of homologous recombination at each targeting step was sufficiently high (0.4–6.4%) to produce at least a couple of targeted colonies from ~500 selected colonies that were screened by PCR in each experiment. The efficiency might be attributed to several conditions that were optimized specifically for bovine fibroblast targeting, including using appropriate promoters to maximize expression of positive selection marker genes, using the DT-A gene for negative selection[19], using contiguous regions of homology in the targeted gene loci, optimizing electroporation conditions[5] and cloning immediately after PCR selection with a modified system to facilitate reprogramming of the donor cells[20].

Using this sequential targeting strategy, complex genetic modifications, in large animal species, are not only feasible but relatively straightforward and should be useful for many applications. Targeting of multiple genes in large animals may be useful for producing new models for human disease, for producing various therapeutic proteins, for producing organs or tissues for transplantation into humans and for improving the efficiency of agricultural production. Gene targeting has many useful applications in science, medicine and industry and may be one of the most useful applications of somatic cell cloning technology. Currently, gene targeting using ES cells has been successful only in mice, but somatic cell cloning has been successful for many species[21–25]. The results obtained in this study indicate that complex genetic modifications can now be readily made for a wide variety of genes in many species.

## METHODS

**Constructing knockout vectors.** We obtained a bovine genomic fragment around exon 2 of the *IGHM* constant region locus from nonisogenic Holstein genomic library by probing with a $^{32}$P-labeled PCR fragment. We analyzed one genomic clone further by restriction mapping. We subcloned 7.2 kb of the *Bgl*II-*Xho*I genomic fragment (5′ homologous arm) and 2.0 kb of the *Bam*HI-*Bgl*II fragment (3′ homologous arm) around exon 2 into pBluescript II SK(-) (Stratagene) and then inserted *puro*, STOP cassettes (pBS302, Stratagene) and DT-A genes (pBCμΔKOpuro vector). To construct the second targeting vector, we carried out genomic PCR on cell line 6939. After digestion with *Bam*HI-*Bgl*II, this fragment replaced the 3′ short arm of the pBCμΔKOpuro vector. By sequencing, we confirmed that the *Bam*HI-*Bgl*II fragment was amplified from allele A. We replaced the *puro* gene with a *neo* gene (pBCμΔNKOneo vector). We obtained bovine genomic fragment around exon 3 of *PRNP* locus by screening the same Holstein genomic λ phage library with a $^{32}$P-labeled DNA fragment amplified by PCR. We analyzed one genomic clone further by restriction mapping. We subcloned 8.3 kb of the *Bam*HI genomic fragment (3′ homologous arm) and 1.2 kb of the *Bam*HI-*Bgl*II fragment (5′ homologous arm) containing exon 3 into pBluescript II SK(-) and inserted both *neo* and STOP cassettes at the *Bam*HI site, which is behind the initial ATG codon. We also subcloned the DT-A gene (pBPrP(H)KOneo vector). Similarly, we constructed another knockout vector containing the *puro* gene (pBPrP(H)KOpuro vector). Primer sequences are available on request.

**Cell culture and transfection.** We cultured Holstein fetal male fibroblasts as previously described[26] and electroporated them with 30 μg of each targeting vector at 550 V and 50 μF by using a GenePulser II (Bio-rad). After 48 h, we selected the cells under 500 μg ml$^{-1}$ of G418 or 1 μg ml$^{-1}$ of puromycin for

2 weeks, picked the drug-resistant colonies and transferred them to replica plates, one for genomic DNA extraction (24-well plates) and the other for embryonic cloning (48-well plates).

**Genomic PCR analyses.** From the replica 24-well plates, we extracted fetus or ear biopsy genomic DNA from calves using a Puregene DNA extraction kit (GentraSystem). To identify each homologous recombination event that occurred at the *IGHM* locus, we used primer pairs puroF2, puroR2, neoF3 and neoR3 (Fig. 2a). PCR was done in 30 cycles of 98 °C for 10 s and 68 °C for 8 min. For negative PCR, we used primer pairs BCμf and BCμr (Fig. 2a) in 40 cycles of PCR composed of 98 °C for 10 s, 62 °C for 30 s and 72 °C for 1 min. In the case of the *PRNP* locus, we used primer pairs neoF7, neoR7, puroF14 and puroR14 (Fig. 4a). PCR was done in 30 cycles of 98 °C for 10 s and 68 °C for 5 min. For negative PCR, we used primer pairs BPrPexF and BPrPexR (Fig. 4a) in 40 cycles of PCR composed of 98 °C for 10 s, 62 °C for 30 s and 72 °C for 1 min. To detect the Cre-mediated excision, we carried out PCR with primer pair CreExF and CreExR (Fig. 3a) in 40 cycles of PCR composed of 98 °C for 10 s and 68 °C for 7 min. All the PCR products were separated on 0.8% agarose gels. Primer sequences are available on request.

**Sequencing analysis of the PCR products.** To confirm whether homologous recombination correctly occurred at each targeting step, we sequenced the amplified PCR products. We purified the PCR products through CHROMA SPIN-TE400 column (BD Biosciences Clontech) and sent them to ACGT for sequencing. Bidirectional sequencing was done with both the forward and reverse primers that were used for PCR. The allele into which each knockout vector was integrated was determined by polymorphisms in the sequence of the PCR products.

**Embryonic cloning.** We produced cloned fetuses and calves as described previously[20]. We enucleated *in vitro* matured oocytes 20 h after maturation. We permeabilized correctly targeted clones by incubating ~50–100,000 cells in suspension with 31.2 U Streptolysin O (Sigma) in 100 μl of Hank's balanced salt solution for 30 min in a water bath at 37 °C. Permeabilized cells were sedimented, washed and incubated with 40 μl of mitotic extract containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate and 25 μg $ml^{-1}$ of creatine kinase) for 30 min at 38 °C. At the end of the incubation, we diluted the reaction mix, sedimented the cells and washed them. We fused these cells to enucleated oocytes, activated 28 h after maturation with 5 μM calcium ionophore for 4 min followed by 10 μg $ml^{-1}$ of cycloheximide and 2.5 μg $ml^{-1}$ of cytochalasin D for 5 h. After activation, we washed the embryos and cultured them with mouse fetal fibroblasts to the blastocyst stage *in vitro*. We selected grade 1 and 2 blastocysts and transferred them into synchronized recipients. All animal work was done following a protocol approved by the Transova Genetics Institutional Animal Care and Use Committee.

**RT-PCR.** We extracted RNA from spleens of wild-type (6939) and *IGHM*$^{-/-}$ fetuses using an RNeasy mini kit (Qiagen) and carried out first-strand cDNA synthesis using the Superscript first-strand synthesis system for RT-PCR (Invitrogen). We carried out PCR using primers BCμf and BCμr in 40 cycles composed of 98 °C for 10 s, 62 °C for 30 s and 72 °C for 1 min. We also extracted RNA from 4658 (*IGHM*$^{-/-}$), 8443 (*IGHM*$^{-/-}$ *PRNP*$^{+/-}$) and doubly homozygous knockout (*IGHM*$^{-/-}$ *PRNP*$^{-/-}$) fibroblasts and carried out first-strand cDNA synthesis as above. PCR was done using primers PrPmF3 and PrPmR3 in 40 cycles of 98 °C for 10 s, 62 °C for 30 s and 72 °C for 1 min. To detect expression of bovine β-actin mRNA, we used primers bBAF and bBAR in the same PCR condition (data not shown). To exclude the possibility of genomic DNA contamination, we carried out another RT-PCR without reverse transcriptase (data not shown). The PCR products were separated on 0.8% agarose gel. Primer sequences are available on request.

ACKNOWLEDGMENTS

We thank J. Pommer, J. Koster, J. Molina and D. Faber for their assistance in embryo transfer, fetal recovery, calf delivery and sample collection and M. Nichols, J. Griffin, M. Bien, T. King, M. Ahlers, R. Paulson, S. Viet and C. Voss for their assistance in gene targeting and embryo cloning.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

Received 3 March; accepted 22 April 2004
Published online at http://www.nature.com/naturegenetics/

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BIOLOGY OF REPRODUCTION 69, 437–445 (2003)
Published online before print 2 April 2003.
DOI 10.1095/biolreprod.102.014647

# Production of α1,3-Galactosyltransferase-Knockout Cloned Pigs Expressing Human α1,2-Fucosylosyltransferase[1]

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## ABSTRACT

**The production of genetically engineered pigs as xenotransplant donors aims to solve the severe shortage of organs for transplantation in humans. The first barrier to successful xenotransplantation is hyperacute rejection (HAR). HAR is a rapid and massive humoral immune response directed against the pig carbohydrate Galα1,3-Gal epitope, which is synthesized by α1,3-galactosyltransferase (α1,3-GT). The Galα1,3-Gal antigen also contributes to subsequent acute vascular rejection events. Genetic modifications of donor pigs transgenic for human complement regulatory proteins or different glycosyltransferases to downregulate Galα1,3-Gal expression have been shown to significantly delay xenograft rejection. However, the complete removal of the Galα1,3-Gal antigen is the most attractive option. In this study, the 5′ end of the α1,3-GT gene was efficiently targeted with a nonisogenic DNA construct containing predominantly intron sequences and a Kozak translation initiation site to initiate translation of the neomycin resistance reporter gene. We developed two novel polymerase chain reaction screening methods to detect and confirm the targeted G418-resistant clones. This is the first study to use Southern blot analysis to demonstrate the disruption of the α1,3-GT gene in somatic HT-transgenic pig cells before they were used for nuclear transfer. Transgenic male pigs were produced that possess an α1,3-GT knockout allele and express a randomly inserted human α1,2-fucosylosyltransferase (HT) transgene. The generation of homozygous α1,3-GT knockout pigs with the HT-transgenic background is underway and will be unique. This approach intends to combine the α1,3-GT knockout genotype with a ubiquitously expressed fucosyltransferase transgene producing the universally tolerated H antigen. This approach may prove to be more effective than the null phenotype alone in overcoming HAR and delayed xenograft rejection.**

*assisted reproductive technology, embryo, gamete biology, gene regulation, oocyte development*

## INTRODUCTION

The promise of an unlimited supply of suitable pig cells, tissues, and organs for transplantation into humans is the impetus for continuing research efforts to develop a universally accepted animal source for xenotransplantation. Despite formidable obstacles and the slow rate of progress, advances in technology and genetic modification of animals can make xenotransplantation a reality [1, 2]. Discordant pig xenografts are rejected by humoral and cellular immune responses [1]. Hyperacute rejection (HAR) represents the first major immunological hurdle encountered by the xenograft and occurs within minutes to hours after transplantation [3]. HAR is an aggressive response mediated by natural antibody reactivity directed against the pig carbohydrate Galα1,3-Gal epitope followed by complement activation [4]. When HAR is averted in vascularized organs by plasmapheresis [5] or complement inhibition [6], the xenograft is still subject to delayed xenograft rejection (DXR). DXR is characterized by a strong humoral response, type II endothelial cell activation, and an acute cellular infiltrate [1]. In avascular tissues such as cartilage, the delayed rejection process also involves a strong antibody response and a cellular immune infiltrate [7]. Galα1,3-Gal antigen plays a relevant role in these delayed rejection events [7]. The Galα1,3-Gal epitope is synthesized by α1,3-galactosyltransferase (α1,3-GT), which adds a terminal galactose to the acceptor substrate N-acetyl lactosamine [8].

A number of laboratories are attempting to produce α1,3-GT knockout pigs, and two have recently reported success using primary fibroblasts on a wild-type (WT) background [9–11]. However, it is unclear whether the resulting carbohydrate phenotype will be innocuous in the transplant setting. The combination of the α1,3-GT knockout with other approaches aimed at terminally glycosylating an uncapped N-acetyl lactosamine residue may be more effective in overcoming carbohydrate-mediated rejection events. The Galα1,3-Gal epitope can be downregulated by competition between α1,3-GT and α1,2-fucosylosyltransferase (HT) for the common acceptor substrate N-acetyl lactosamine [12]. HT generates the universally tolerated H antigen [12]. Our group and others have previously shown that cells from HT-expressing mice [13–15] and pigs [16] displayed a significantly reduced expression of the Galα1,3-Gal epitope. This modification resulted in a reduction in xenogenic natural antibody reactivity and an increased resistance to human serum-mediated cytolysis. A similar effect was observed when pig cells were genetically modified with other glycosyltransferases such as α2,3-sialyltransferase [17] or acetylglucosaminyltransferase III [18]. Our study combines these approaches by producing α1,3-GT knockout pigs on an HT-transgenic background. Pigs with the α1,3-GT null phenotype with and without the HT background can be used to determine the terminal glycosylation patterns that are most beneficial for prolonging xenograft survival.

The pig α1,3-GT gene has been isolated [19] and

[1]This work was supported by a grant from The National Institute of Standards and Technology.
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Received: 16 December 2002.
First decision: 17 January 2003.
Accepted: 18 March 2003.

ISSN: 0006-3363. http://www.biolreprod.org

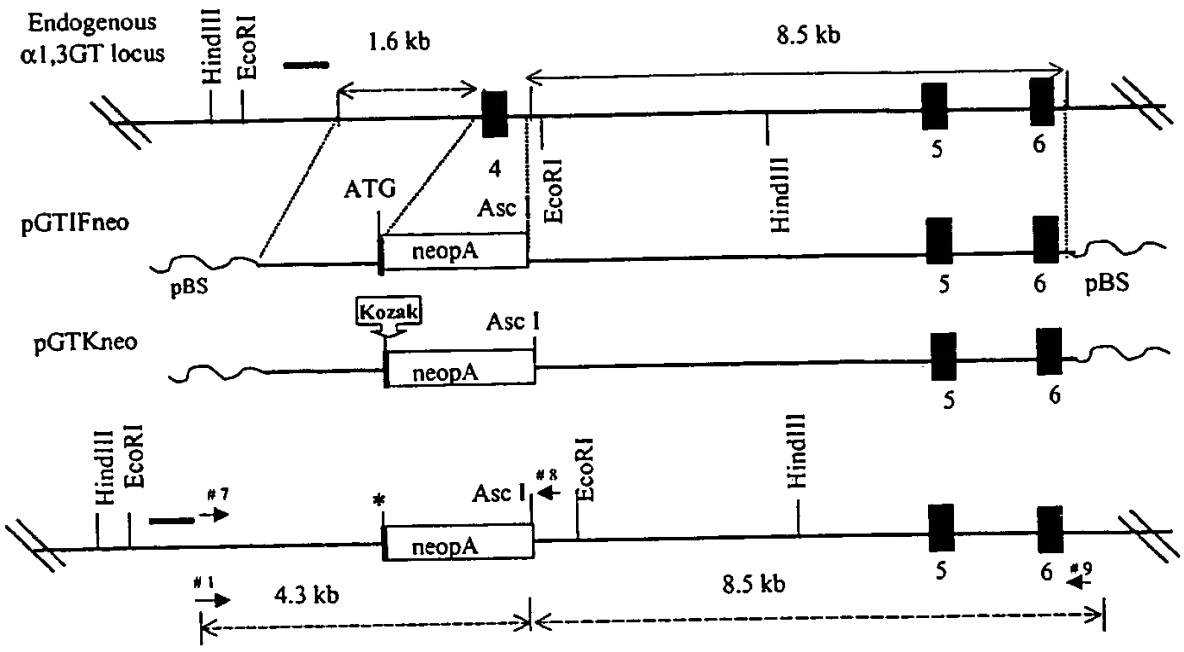


FIG. 1. Design of targeting constructs, and PCR and Southern blot screening strategies. The 5′ end of an endogenous α1,3-GT allele (exons, solid boxes; introns, thick line) showing the regions of homology used for the 5′ and 3′ arms of the targeting constructs, the position of the probe (closed bar), and the *Hin*dIII and *Eco*RI restriction enzyme sites used for Southern blot analyses. Targeting constructs pGTIFneo and pGTKneo are shown with the promoterless neo poly(A) (open box) cloned in frame with the endogenous ATG or with a Kozak consensus sequence (open arrow), respectively. A targeted allele shows the position of primers (solid arrowheads) used for PCR analyses and the unique *Asc*I site; * indicates in-frame or Kozak consensus sequence. Primers 7 and 8 amplified 3.4-kb and 2.7-kb targeted and endogenous bands, respectively. Primers 1 and 9 amplified 12.8-kb and 12.1-kb targeted and endogenous bands, respectively.

mapped to chromosome 1 region q2.10-q2.11 as a single-copy gene [20]. The number of exons and exon-intron boundries are completely conserved between the pig and the mouse, but the sizes of the introns are variable and tend to be larger in the pig [19]. Exon 4 contains the endogenous ATG translation initiation codon and encodes the transmembrane domain of the α1,3-GT enzyme [18]. The open reading frame begins within the first 10 bases of exon 4, making this gene a good candidate for gene targeting with a replacement-type construct at its 5′ end. Targeting of the 5′ end of this gene can be accomplished without the generation of fusion proteins or the use of complex internal ribosome entry site (IRES) sequences, whose functionality has positional limitations [21]. However, the difficulty of targeting the 5′ end of the α1,3-GT gene is that exons 4–8 are relatively small, ranging from 34 base pairs (bp) to 139 bp, and are separated by large introns ranging from 2 kilobases (kb) to >7 kb in length. Our 5′ targeting constructs mainly comprise intron nucleotide sequences and introduce a neomycin resistance gene to replace exon 4 at the endogenous ATG. Because intron sequences are more likely than exons to harbor spontaneous mutations, the efficiency of homologous recombination may be affected when using nonisogenic DNA [22].

Here we report the production of α1,3-GT knockout, HT-transgenic pigs. We found that promoter-trap targeting constructs consisting mainly of intron sequence and a Kozak consensus sequence to initiate translation can be used to target genes with characteristics similar to that of α1,3-GT, where the ATG start codon is located in a downstream exon and the exons are relatively small and separated by large introns. We used nonisogenic DNA in preparing the α1,3-GT gene targeting constructs and developed two efficient and accurate polymerase chain reaction (PCR) strategies to detect targeted clones. These pigs are to be used for studies aimed at overcoming the immune rejection responses usually generated by pig xenografts.

## MATERIALS AND METHODS

### *Oocyte and Fetal Fibroblast Collection*

Experiments were conducted under an animal use protocol approved by the Institutional Animal Care and Use Committee of Columbus Farming Corporation (CFC, Sherburne, NY). Oocytes from three different sources were used. One group of oocytes was flushed from the oviducts of superovulated gilts as described previously [23]. Cycling gilts were synchronized by oral administration of altrenogest (Regu-Mate; Inervet, Millsborough, DE), 18 mg/day for 5–9 days depending on the stage of the estrous cycle, and superovulation was induced by a single injection of eCG (Diosynth, Des Plaines, IL) followed by hCG (Inervet) 76 h later. Unfertilized oocytes were collected by retrograde flushing of the oviduct 45–46 h after the hCG injection. A second group of oocytes was obtained from the ovaries of gilts that received the same hormonal treatment except that ovulation was not induced with the administration of hCG. The gilts were killed 73 h after eCG treatment, their ovaries were removed, and the oocyte-cumulus complexes were collected following slicing of the 3- to 5-mm follicles. These complexes were matured in BSA-free NCSU-23 medium [24] supplemented with 10% porcine follicular fluid, 10 IU/ml eCG, 10 IU/ml hCG, 0.1 mg/ml cysteine, and 10 ng/ml epidermal growth factor. After being cultured for 22 h at 39°C under 5% $CO_2$ in air, the oocyte-cumulus complexes were transferred to fresh medium without hormonal supplementation for an additional 22 h. A third group of oocytes recovered from slaughterhouse pig ovaries was purchased from BoMed (Madison, WI) and shipped to our laboratory in their commercial maturation medium.

A heterozygous transgenic boar expressing the human H-transferase gene [16] was used to breed one cycling gilt (second estrus). The gilt was killed at Day 30 of pregnancy, and 12 fetuses were collected within their amniotic sacs to maintain sterility. Each fetus was processed separately. The head, limbs, and viscera were dissected away, and the remaining tissue was minced into approximately 1-mm pieces. Tissue pieces from each fetus were cultured for fibroblast outgrowth in 25-$mm^2$ (T-25) tissue culture flasks in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 15% fetal calf serum (FCS; Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Genomic DNA was isolated from each fetus and analyzed by PCR for the presence of the H-transferase transgene as previously described [23]. Confluent T-25 flasks were trypsinized, and cells were transferred to T-75 flasks (passage 1), grown to 80–90% confluence, and then frozen in liquid nitrogen for future use. Approximately $2 \times 10^6$ cells/vial in culture medium containing 10% dimethyl sulfoxide (Sigma, St. Louis, MO) were frozen overnight in a −80°C freezer and subsequently stored in liquid nitrogen until use.

### *α1,3-GT Targeting Constructs*

Two targeting constructs were generated to target the α1,3-GT gene (Fig. 1). In targeting construct pGTIFneo, a neomycin-bovine growth hormone poly(A) (neo) sequence cassette was inserted adjacent to and in frame with the endogenous ATG start codon (bases 10–12 of exon 4). The following 471 bases were deleted, which included the remaining 80 bases of exon 4 and 390 bases of intron 4 within the targeting construct. For generation of targeting construct pGTKneo, the neo cassette with a Kozak concensus sequence for the start of translation was used to replace the endogenous ATG, exon 4, and 390 bp of intron 4. An 18-kb DNA fragment from intron 3 to intron 7 was isolated from the EMBL3 porcine

TABLE 1. PCR primers used in the generation of α1,3-GT knockout pigs.

| Primer[a] | Sequence |
|---|---|
| 1-F1765 | TCCATGAACAACTTCGATTGC |
| 2-R14158 | TCCTTCCAGACCAGCACAGTTAGC |
| 3-F3182 | AGTTTCCAACTACTACACTGACTTGC |
| 4-R4856 | ATCTGCAGTATTTTCTCCTGGGAAAAGAAAAGGAGAAGG |
| 5-F5327 | ATCGGCCGTGTATCCCTGAGCCCTTAAATACCG |
| 6-R13831 | ATGGCCGTTTCCCAGTCTTACC |
| 7-F2725 | TTGAGCCAGGCCACCTCCTCTTTATG |
| 8-R5424 | ACAATGGCAACATGGCAGGAAGGAAG |
| 9-R13915 | AAGGACGAGGCGACTGAGAAGGTCATGG |
| 10-F2744 | CTTTATGGTCATGAGAACG |
| 11-R3055 | TGGAAACAGCATCTATACC |

[a] F, forward primer; R, reverse primer. Primer ID numbers represent actual positions of the primers within the 18-kb DNA fragment isolated from the porcine EMBL3 genomic library.

genomic library (BD Biosciences, Clontech, Palo Alto, CA) using an intron 3 fragment as probe for Southern blot screening. The 18-kb fragment was subcloned and sequenced in its entirety and used to design PCR primers (Table 1). DNA from a boar (different from the one used as sire for the fetuses) was used to amplify a 12.4-kb fragment from intron 3 to intron 6 by long-range PCR. Amplification was performed using the Expand Long Template PCR kit (Roche, Indianapolis, IN) with forward primer 1 and reverse primer 2 (Table 1). The cycling conditions were 94°C for 2 min, followed by 94°C for 15 sec, 60°C for 30 sec, and 68°C for 10 min for 10 cycles, 94°C for 15 sec, 60°C for 30 sec, and 68°C for 10 min + 20 sec per cycle for 20 cycles, and a final extension step of 68°C for 8 min. The PCR fragment was cloned into the pCR2.1 cloning vector (Invitrogen) and used as a template to amplify the 5′ and 3′ arms of the constructs. A 1.6-kb fragment was amplified using forward primer 3 and reverse primer 4 and used for the 5′ arm of the constructs. An 8.5-kb fragment was generated and used for the 3′ arm of the constructs using forward primer 5 and reverse primer 6 (Table 1). Except for the extension times, the PCR cycling conditions used were the same as described above. The pBluescript cloning vector (Stratagene, La Jolla, CA) was used to construct the targeting vectors. An *Asc*I restriction enzyme site was placed immediately after the neo poly(A) sequence in both pGTIFneo and pGTKneo constructs. The construct vectors were each transfected into XL10-competent cells (Stratagene), single colonies were amplified, and plasmid DNA isolated by cesium chloride gradient ultracentrifugation. Targeting constructs were linearized with *Not*I outside the region of homology and used for electroporation into primary fibroblasts.

## Fibroblast Transfection Culture and Drug Selection

One transfection was carried out with targeting construct pGTIFneo using fibroblasts from a non-HT-transgenic fetus. Four transfections were carried out with construct pGTKneo using fibroblasts from both HT-transgenic and nontransgenic fetuses. Fetal fibroblasts frozen at passage 2 were thawed and cultured in a T-75 tissue culture flask for 24 h prior to electroporation in DMEM supplemented with 10% FCS, 1% penicillin and streptomycin, 5% minimal essential medium, and 2 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN) at 38°C in an atmosphere of 5% $CO_2$ in air and 100% humidity. Fibroblasts were harvested by trypsinization, and approximately $2 \times 10^6$ cells were electroporated with 10 μg of linearized DNA at 450 V and 350 μF using a Gene Pulser II electroporator (Bio-Rad Laboratories, Hercules, CA). Electroporated cells were cultured for 48 h before transfer into 96-well plates ($2 \times 10^3$ cells/well) or 100-mm tissue culture plates ($2 \times 10^5$ cells/plate) for selection in culture medium containing G418 (Geneticin, 600 μg/ml; Invitrogen). Selection was carried out for 10 days, and wells with resistant colonies were each transferred in triplicate to 96-well plates. Wells from one plate were used for DNA isolation and PCR analysis of targeted clones. Possible targets from the second plate were expanded for Southern blot analysis, and cells from the third plate were expanded and frozen as described above in small aliquots ($10^5$ cells/vial) for nuclear transfer (at approximately 20 cell doublings from the single cell targeting event). G418-resistant colonies from 100-mm plates were transferred by the cloning ring method to 24-well tissue culture plates for expansion and analysis.

## PCR and Southern Blot Analyses of Neomycin-Resistant Colonies

DNA was isolated from G418-resistant colonies in 96-well plates by lysis in TE buffer (50 mM Tris, pH 8, 2.5 mM EDTA, 100 mM NaCl, and 0.1% SDS) for 3 h at 60°C followed by isopropanol precipitation, and pellets were washed twice in 70% ethanol and resuspended in 30 μl TE. Preliminary screening for targeted events was carried out by PCR using two sets of primers. The first pair of primers flanked the 5′ arm of the construct and the neomycin resistance gene cassette and amplified targeted and endogenous bands of 3.4 kb and 2.7 kb, respectively, in single-allele α1,3-GT knockout clones. The primers used were forward 7 and reverse 8 (Table 1). The LA *Taq* PCR kit (Takara; Panvera Corporation, Madison, WI) was used, with 2 μl of DNA from each G418-resistant colony as template. The cycling conditions were 94°C for 2 min, followed by 94°C for 15 sec, 65°C for 30 sec, and 72°C for 3 min for 10 cycles, 94°C for 15 sec, 65°C for 30 sec, and 72°C for 3 min + 20 sec per cycle for 20 cycles, and a final extension step of 72°C for 8 min.

For confirmation, long-range PCR was carried out on potential targeted clones using a second pair of primers that bind to sequences outside both arms of the targeting construct within the flanking chromosomal DNA. Both targeted and endogenous fragments of 12.8 kb and 12.1 kb were amplified, respectively. The LA *Taq* PCR kit was used, with 5 μl of DNA from each G418-resistant colony as template. Forward primer 1 and reverse primer 9 (Table 1) were used. The cycling conditions were 94°C for 2 min, followed by 94°C for 15 sec, 65°C for 30 sec, and 68°C for 10 min for 10 cycles, 94°C for 15 sec, 65°C for 30 sec, and 68°C for 10 min + 20 sec per cycle for 20 cycles, and a final extension step of 68°C for 8 min. The amplified DNA fragments were large and too close in size to be separated, but when cut with *Asc*I the 12.8-kb fragment amplified from the targeted allele produced two pieces, 8.5 kb and 4.3 kb, that were clearly distinguishable from the endogenous 12.1-kb band after electrophoresis on a 0.7% agarose gel.

Genomic DNA isolated from cells indicated as targeted by PCR and from fetuses and piglets following nuclear transfer was analyzed by Southern blotting [25] following standard procedures. For Southern blotting, DNA was digested with *Eco*RI or *Hin*dIII (New England Biolabs, Beverly, MA), and approximately 2 μg and 5 μg from cells and tissues, respectively, were separated on 0.7% agarose gels and blotted to Hybond-N+ membrane (Amersham, Pharmacia Biotech, Piscataway, NJ). A 311-bp DNA probe corresponding to intron 3 of the α1,3-GT gene just outside the 5′ end of the targeting construct was produced by PCR using forward primer 10 and reverse primer 11 (Table 1). The probe was labeled using [α-$^{32}$P]dCTP (Amersham) by random oligopriming (Stratagene). Membranes were subsequently hybridized overnight at 65°C with the radiolabeled probe in a hybridization solution containing Denhardt reagent, washed with increasing levels of stringency, and exposed to Kodak XAR-5 film (Sigma).

## Nuclear Transfer

Oocytes were manipulated at 46–48 h after hCG for ovulated oocytes and 40–42 h after initiation of maturition for in vitro-matured oocytes. Except for minor modifications, the procedure was as previously described [23]. For enucleation, the medium was supplemented with 1% and 5% sucrose for ovulated and in vitro-matured oocytes, respectively. The reconstructed oocytes were equilibrated in Ca-free fusion medium (pH 7.1; containing 300 mM mannitol, 0.2 mM $MgSO_4$, 0.5 mM Hepes, 0.1% polyvinyl alcohol) for 5 min, and then each oocyte was placed between the two electrodes of a fusion chamber (BTX, San Diego, CA) overlaid with fusion medium. Fusion was induced with two DC pulses of 1.2 kV/cm for 60 μsec each. Two minutes after the electrical pulse, the reconstructed oocytes were transferred into Beltsville embryo culture medium (BECM) and cultured for 1 h. They were then activated by two DC pulses

TABLE 2. Results of analysis of G418-resistant colonies from five independent transfections of primary fetal fibroblasts for the generation of α1,3-GT knockout pigs.

| Fibroblast cell line | Targeting construct | No. G418-resistant colonies | No. (%) PCR-positive colonies | | No. (%) Southern blot-positive colonies |
|---|---|---|---|---|---|
| | | | Preliminary PCR | Long range PCR | |
| $C2.1^{HT-}$ | pGTIFneo | 217 | 2 (0.9) | 2 (0.9) | 2 (0.9) |
| $F1.1^{HT+}$ | PGTKneo | 299 | 4 (1.3) | 4 (1.3) | 4 (1.3) |
| $F1.2^{HT+}$ | PGTKneo | 656 | 9 (1.37) | 8 (1.2) | 8 (1.2) |
| $C2.0^{HT-a}$ | PGTKneo | 54 | 4 (7.4) | 4 (7.4) | ND |
| $F1.0^{HT+a}$ | PGTKneo | 46 | 4 (8.7) | 4 (8.7) | ND |

[a] G418-resistant colonies isolated by the cloning ring method from 100-mm tissue culture plates. Southern blots were not done (ND).

as described for fusion except that the medium (pH 7.1) overlaying the fusion chamber contained 300 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM Hepes, and 0.1% polyvinyl alcohol. Nuclear transfer embryos were either transferred into recipient animals the same day or, in some cases to obtain a higher number of embryos for transfer, were cultured overnight in NCSU-23 medium before transfer. These embryos were then pooled with nuclear transfer embryos produced the following day. In this study, a total of 2918 nuclear transfer embryos were transferred to 20 recipient gilts (mean, 146 embryos/recipient; range, 97–246). Recipients were synchronized by oral administration of altrenogest (18 mg/day for 5–9 days depending on the stage of the estrous cycle) and stimulated with 500 IU hCG 72 h later. The synchrony of the recipients' estrous cycles was delayed by 24–48 h from that of the oocyte donors. Reconstructed embryos were transferred approximately 32 h after hCG injection, at which time the recipient animals had not yet ovulated.

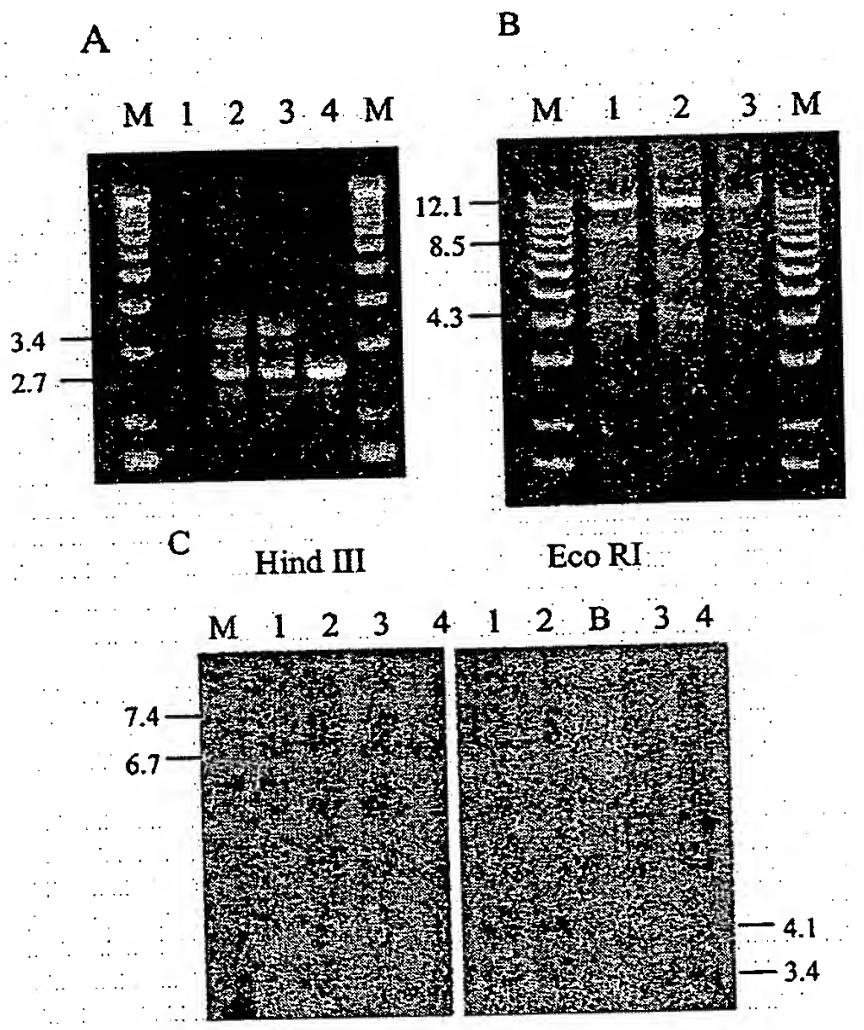


FIG. 2. PCR and Southern blot analyses of G418-resistant clones transfected with the pGTIFneo targeting construct. A) Preliminary PCR. Lane M: 1-kb DNA marker; lane 1: no DNA template control; lane 2: $C172^{HT-}$; lane 3: $C173^{HT-}$; lane 4: $C2.1^{HT-}$ cell line (nontransfected control). Primers 7 and 8 amplified 3.4-kb and 2.7-kb targeted and endogenous bands, respectively. B) Long-range PCR. Lane M: 1-kb DNA marker; lane 1: $C172^{HT-}$; lane 2: $C173^{HT-}$; lane 3: $C2.1^{HT-}$ cell line control. Primers 1 and 9 amplified a 12.8-kb targeted band (*Asc*I cut, 8.5-kb and 4.3-kb) and a 12.1-kb endogenous band. C) Southern blot analysis with *Hind*III- and *Eco*RI-digested DNA. First panel: *Hind* III-cut DNA. Lane M: 1-kb DNA marker; lane 1: $C172^{HT-}$; lane 2: $C173^{HT-}$; lane 3: $C2.1^{HT-}$ nontransfected cell line control; lane 4: control pig DNA. Second panel: *Eco*RI-cut DNA. Lane 1: $C172^{HT-}$; lane 2: $C173^{HT-}$; lane B: blank; lane 3: $C2.1^{HT-}$ nontransfected cell line control; lane 4: control pig DNA. The 7.4-kb targeted and 6.7-kb endogenous bands were produced with *Hind*III, and the 4.1-kb targeted and 3.4-kb endogenous bands were produced with *Eco*RI.

### *Flow Cytometric Analysis*

Expression of the α1,3-GT gene and the HT transgene was assessed by flow cytometry of primary cultured fibroblasts. Direct fluorescence of cell-surface carbohydrate epitopes was performed with fluorescein isothiocyanate-conjugated lectins as described previously [16, 23].

## RESULTS

The α1,3-GT enzyme is expressed in fetal and adult pig fibroblasts, and the Galα1,3-Gal antigen is readily detected on the cell surface; therefore, the use of a promoter-trap replacement type targeting strategy was appropriate. The targeting constructs used here are homologous to sequences at the 5′ end of the α1,3-GT gene. The ATG translation start codon located at the 5′ end of exon 4 (bases 10–12) was used as the start codon for the neomycin resistance gene in the targeting construct pGTIFneo. A second construct, pGTKneo, utilized a synthetic Kozak consensus sequence and ATG to initiate translation of the neomycin resistance gene. These replacement-type constructs contained 10.1 kb of sequence homology comprising an 8.5-kb long 3′ arm and a short 1.6-kb 5′arm. The positions of primers and the DNA probe sequence used for target analyses are indicated in Figure 1. A total of five independent electroporations were performed with primary fetal fibroblast cell lines isolated from two different fetuses (fetus F, $HT^+$; fetus C, nontransgenic; passages 2 and 3). One cell line was transfected with construct pGTIFneo, and four were transfected with pGTKneo. Table 2 gives a summary of the results obtained.

### *Gene Targeting with pGTIFneo*

The cell line $C2.1^{HT-}$ isolated from nontransgenic fetus C was electroporated with pGTIFneo, and 217 G418-resistant colonies (Table 2) were analyzed for successfully targeted events using primers 7 and 8. Two colonies, $C17D2^{HT-}$ and $C17D3^{HT-}$, showed the 3.4-kb and 2.7-kb targeted and endogenous bands, respectively (Fig. 2A). Targeting was confirmed using primers 1 and 9 flanking the entire construct (Fig. 2B). The targeted band was cut with *Asc*I to get the 8.5- and 4.3-kb fragments indicative of a targeting event. The 12.1-kb endogenous band was uncut (Fig. 2B). Southern blot analysis of DNA from clones $C17D2^{HT-}$ and $C17D3^{HT-}$ and from the nontransfected $C2.1^{HT-}$ parental cell line digested with *Hind*III or *Eco*RI revealed the 7.4-kb and 4.1-kb targeted bands and 6.7-kb and 3.4-kb WT bands, respectively (Fig. 2C). A 0.9% rate

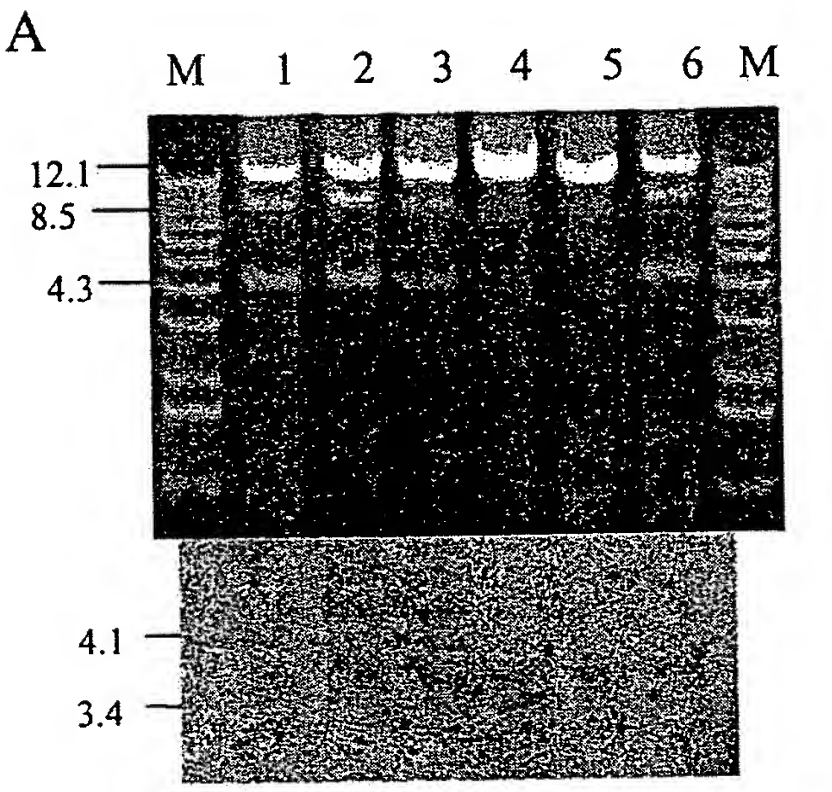


FIG. 3. PCR and Southern blot analyses of G418-resistant clones transfected with the pGTKneo targeting construct. A) Long-range PCR (*Asc*I-digested product, top) and Southern blot (*Eco*RI-digested DNA, bottom) analyses of G418-resistant clones of F1.1$^{HT+}$ fibroblasts transfected with pGTkneo. Lane M: marker; lane 1: F27$^{HT+}$; lane 2: NF28$^{HT+}$; lane 3: NF29$^{HT+}$; lane 4: F1.3$^{HT+}$ cell line nontransfected control; lane 5: control pig DNA; lane 6: F210$^{HT+}$. B) Long-range PCR (top) and Southern blot (bottom) analyses of G418-resistant clones of F1.2$^{HT+}$ fibroblasts transfected with pGTkneo (*Eco*RI-digested DNA). Lane M: marker; lane 1: F13$^{HT+}$; lane 2: F23$^{HT+}$; lane 3: F54$^{HT+}$; lane 4: F62$^{HT+}$; lane 5: F612$^{HT+}$; lane 6: F93$^{HT+}$; lane 7: F71$^{HT+}$; lane 8: F72$^{HT+}$; lane 9: F129$^{HT+}$; lane 10: F3.1$^{HT+}$ cell line nontransfected control. Long-range PCR primers 1 and 9 amplified 12.8-kb (*Asc*I digested, 8.5 kb and 4.3 kb) and 12.1-kb endogenous bands. For Southern blot, 4.1-kb targeted and 3.4-kb endogenous bands were produced with *Eco*RI.

of targeting was observed in all three analyses (Table 2). The C17D3$^{HT-}$ cells were frozen in small aliquots and used as nuclear transfer donors.

### *Gene Targeting with pGTKneo*

Results from targeting with pGTKneo are presented in Table 2 and Figure 3. PCR and Southern blotting were used to analyze a total of 1055 G418-resistant colonies from four transfections. The F1.1$^{HT+}$ cell line was used in the first transfection, and 299 drug-resistant colonies were analyzed. Four colonies, F27$^{HT+}$, F28$^{HT+}$, F29$^{HT+}$, and F210$^{HT+}$, were detected as targeted by PCR with primers 7 and 8 (data not shown). This targeting was confirmed by long-range PCR with primers 1 and 9 flanking the entire construct, digestion of the PCR product with *Asc*I (Fig. 3A, top), and Southern blot analysis (Fig. 3A, bottom). A 1.3% rate of targeting was determined by all three methods (Table 2). Cells from targeted clones F27$^{HT+}$, F29$^{HT+}$, and F210$^{HT+}$ were used as nuclear transfer donors.

Cell line F1.2$^{HT+}$ was transfected with pGTKneo, and 656 drug-resistant colonies were analyzed (Table 2). PCR using primers 7 and 8 revealed that nine clones (F13$^{HT+}$, F23$^{HT+}$, F54$^{HT+}$, F62$^{HT+}$, F612$^{HT+}$, F93$^{HT+}$, F71$^{HT+}$, F72$^{HT+}$, and F129$^{HT+}$) were targeted (data not shown). When these clones were subjected to long-range PCR analysis using primers 1 and 9 (Fig. 3B, top) and to Southern blot analyses (Fig. 3B, bottom), eight of the nine clones were confirmed as α1,3-GT targeted mutants. Clone F129$^{HT+}$ (Fig. 3B, lane 9) did not show a mutant banding pattern. Two additional transfections with construct pGTKneo were performed using F1.0$^{HT+}$ and C2.0$^{HT-}$ cell lines (Table 2). Tissue culture plates (100 mm) and cloning rings were used for selection of drug-resistant colonies instead of 96-well plates. From C2.0$^{HT-}$ and F1.0$^{HT+}$ transfected cells, 54 and 46 G418-resistant colonies were transferred by the cloning ring method to 24-well plates, respectively. In each case, four targeted clones were identified by PCR (Table 2), to give targeted mutation rates of 7.4% and 8.7%, respectively. Southern blot analysis was not performed on these clones.

TABLE 3. Summary of nuclear transfer results for the generation of α1,3-GT knockout male pigs.

| | Donor cell lines | | | |
|---|---|---|---|---|
| | C173$^{HT-}$ | F210$^{HT+}$ | F27$^{HT+}$ | F29$^{HT+}$ |
| No. oocytes | | | | |
| BoMed | 604 | 483 | 154 | 454 |
| CFC, in vitro matured | 484 | 101 | 214 | 97 |
| CFC, ovulated | 130 | 94 | 55 | 52 |
| No. embryos transferred | 1218 | 674 | 423 | 603 |
| No. recipients | 7 | 4 | 4 | 5 |
| No. pregnant on day 30 | 3 | 1[a] | 1 | 2 |
| No. pregnant on day 60 | 3 | 0 | 0 | 2 |
| No. pregnancies brought to term | 1 | 0 | 0 | 2 |
| No. piglets at birth | 4 | 0 | 0 | 8[b] |
| No. live α1,3-GT knockout$^{HT-}$ pigs | 2 | 0 | 0 | 0 |
| No. live α1,3-GT knockout$^{HT+}$ pigs | 0 | 0 | 0 | 4 |

[a] Pregnancy terminated for fetal fibroblast collection.
[b] The first litter from F29$^{HT+}$ reconstructed embryos consisted of two piglets (live births), and the second litter consisted of six piglets (two live births and four stillbirths).

### *Production of α1,3-GT Knockout Pigs by Nuclear Transfer*

Four different targeted cell lines, C173$^{HT-}$, F210$^{HT+}$, F27$^{HT+}$, and F29$^{HT+}$, were used as donors for nuclear transfer (Table 3). The karyotype of the parent cell lines (before targeting) used in this study appeared normal, with >75% of the chromosome spreads displaying a full complement of 38 chromosomes (data not shown). A total of 2918 nuclear transfer embryos were transferred to 20 recipient gilts (mean, 146 embryos/recipient; range, 97–246). Seven preg-

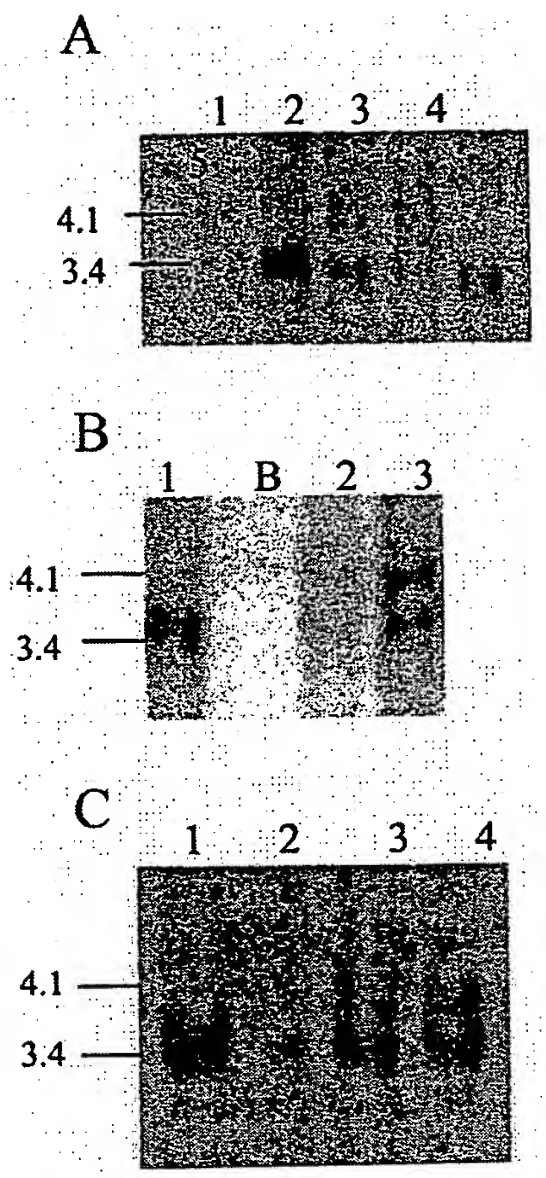


FIG. 4. Southern blot analysis of *Eco*RI-digested DNA from nuclear transfer cloned piglets and fetuses, producing 4.1-kb targeted and 3.4-kb endogenous bands. **A)** Litter 136. Lane 1: piglet 136-1$^{HT-}$; lane 2: piglet 136-2$^{HT+}$; lane 3: piglet 136-3$^{HT-}$; lane 4: piglet 136-4$^{HT-}$; lane 5: recipient, DNA control. **B)** Southern blot analysis of *Eco*RI-digested DNA from litter 141, Lane 1: recipient, DNA control; lane 2: blank; lane 3: piglet 141-1$^{HT+}$; lane 4: piglet 141-2$^{HT+}$. **C)** Southern blot analysis of *Eco*RI-digested DNA from fetuses derived from C173$^{HT-}$ and F210$^{HT+}$ reconstructed embryos. Lane 1: fetus 1 (reabsorbing); lane 2: fetus 2$^{HT-}$; lane 3: fetus 3$^{HT+}$; lane 4: fetus 4$^{HT+}$.

nancies were detected by ultrasonography at Day 30, five were detected at Day 60, and three were carried to term, resulting in eight live births (one piglet died soon after birth) and four stillbirths (Table 3). Figure 4A shows Southern blot analysis of the four piglets cloned from C173$^{HT-}$ and a nontargeted cell line. The 4.1-kb targeted and 3.1-kb endogenous bands were detected in DNA samples isolated from piglets 1, 3, and 4 (Fig. 4A, lanes 1, 3, and 4). Figure 4B shows Southern blot analysis of the two piglets from the first litter cloned from F29$^{HT+}$ cells. The targeted and endogenous bands were found in DNA samples from both piglets (Fig. 4B, lanes 2 and 3). DNA was isolated from each of the six piglets (four stillborn and two live) from the second litter cloned from F29$^{HT+}$ cells and was analyzed by PCR. DNA from all six piglets showed the 3.4-kb targeted and 2.7-kb endogenous bands, using primers 7 and 8 (data not shown). The presence of a mutated allele was confirmed by long-range PCR using primers 1 and 9. A targeted band (digested with *Asc*I to give the 8.5-kb and 4.3-kb targeted bands) from the mutated allele and the 12.1-kb endogenous band were evident in all six piglet samples (data not shown). Figure 4C shows Southern blot analysis of the four fetuses harvested for fibroblasts. The 4.1-kb targeted and 3.1-kb endogenous bands were detected in DNA samples isolated from fetuses 2, 3, and 4. Fetus 1 was reabsorbing, and a targeted band was not evident in the partially degraded DNA (Fig. 4C).

To increase the number of embryos transferred per recipient, some embryos were cultured in NCSU-23 medium for 24 h and then transferred together with nuclear transfer embryos produced the following day. In some cases, nuclear transfer experiments were conducted with nontargeted cells. The cells used in the non-gene targeting experiments were from a different cell line, which produces black piglets instead of the white piglets from the targeted cell lines (see Fig. 5). The fibroblasts used in the non-gene targeting experiments also expressed the HT transgene.

Embryos reconstructed with C173$^{HT-}$ cells were transferred to seven recipients and resulted in three established pregnancies (detected by ultrasonography at Days 30 and 60 of pregnancy). Two recipients aborted after Day 60, and one carried the pregnancy to term, producing a litter of four piglets (Table 3 and Fig. 5A, inset shows litter at birth). This litter resulted from the transfer of targeted and nontargeted nuclear transfer embryos. One of the four piglets born was clearly from the nontargeted (black) cell line. One piglet from the litter died soon after delivery from an undetermined cause, and the remaining three are healthy at 2 mo of age (Fig. 5A).

Three recipients received F210$^{HT+}$ reconstructed embryos, and one received C173$^{HT-}$ and F210$^{HT+}$ reconstructed embryos. A pregnancy resulted from the recipient transferred with the combination of F210$^{HT+}$ and C173$^{HT-}$ reconstructed embryos (Table 3). This recipient was killed at Day 30 of pregnancy, and the fetuses were collected to establish fetal fibroblast cell lines for further experiments. Two of the fetuses developed from C173$^{HT-}$ cells, and the remaining two were from F210$^{HT+}$ cells, as determined by flow cytometry detecting HT expression (data not shown). One of the four transfers with F27$^{HT+}$ reconstructed embryos resulted in a pregnancy that aborted after 30 days (Table 3). Two of the five transfers with F29$^{HT+}$ reconstructed embryos became pregnant and produced litters of two piglets (Fig. 5B) and six piglets (four stillborn and two live; Table 3). Overall, five pregnancies were obtained with 1227 embryos produced from oocytes ovulated (331 oocytes) or in vitro matured (896 oocytes) after collection from pigs kept in our facility at CFC. In comparison, two pregnancies resulted from BoMed in vitro-matured oocytes (1695 oocytes): one aborted after Day 60 and the second produced a litter of two piglets. The first two pregnancies were allowed to proceed to 119 days (5 days past the due date of 114 days), delivery was by caesarian section, and there was no milk let down. The third pregnancy was allowed to go to term naturally, parturition occurred at 116 days, and there was milk let down; however, there were four stillborn piglets and two live piglets.

### *Detection of Cell Surface Expression of H and Galα1,3-Gal Carbohydrate Antigens*

Nuclear transfer using α1,3-GT knockout donor cells derived from HT-transgenic or nontransgenic fetuses successfully produced piglets. The nontransgenic cell line C2.1$^{HT-}$ exhibits a high level of expression of the Galα1,3-Gal epitope but lacks H antigen expression (Fig. 6A). The litter (no. 136) of four piglets was produced from embryos reconstructed using the nontransgenic C173$^{HT-}$ targeted cell

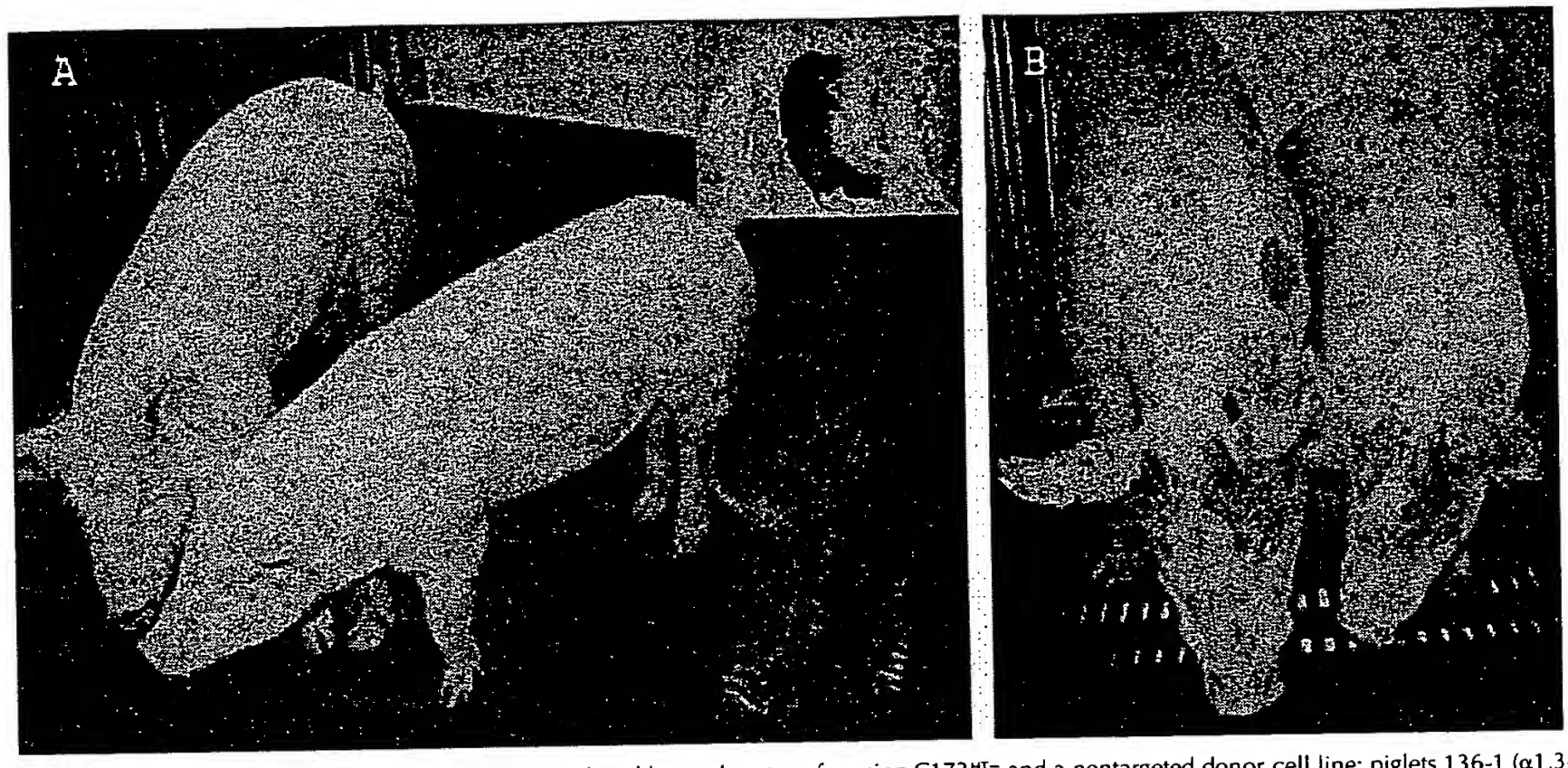


FIG. 5. Nucelar transfer live piglets. A) Litter 136 produced by nuclear transfer using C173$^{HT-}$ and a nontargeted donor cell line: piglets 136-1 (α1,3-GT knockout, white), 136-2 (α1,3-GT WT, black), and 136-4 (α1,3-GT knockout, white). Inset shows the entire litter at birth: three α1,3-GT knockout (white) and one WT (black) piglets. B) Two α1,3-GT knockout piglets from litter 141 cloned by nuclear transfer from cell line NF29$^{HT+}$: piglets 141-1$^{HT+}$ and 141-2$^{HT+}$.

line and from nontargeted cells that express HT (Fig. 6). Three of the piglets, 136-1, 136-3, and 136-4, did not express HT and showed a slight reduction in Galα1,3-Gal epitope expression as compared with the parental cell line (Fig. 6, A and C). However, the first litter (no. 141) of two piglets produced from the F29$^{HT+}$ α1,3-GT targeted cell line expressed both HT and the Galα1,3-Gal epitopes as strongly as did the parental cell line F1.1$^{HT+}$ (Fig. 6, B and D).

## DISCUSSION

Flow cytometry results have indicated that the α1,3-GT gene is expressed in pig fibroblasts, which allowed us to use a promoter-trap targeting strategy. A targeting construct with a modified translation initiation site based on the Kozak consensus sequence (for review see [26]) was used to initiate translation of the neomycin resistance gene. A second construct with the neo gene inserted in frame with the endogenous ATG was also produced. The use of a Kozak consensus sequence allowed for flexibility in designing the targeting construct because 1) the selectable marker gene can be placed under the control of the endogenous promoter without it having to be cloned in frame with the coding sequence, i.e., the 5′ untranslated region of a gene can be targeted with a synthetic Kozak-ATG consensus sequence for translation initiation, 2) there was no need to generate a fusion protein with the selectable marker gene that may have reduced functionality, 3) it does not require the use of IRES sequences, and 4) it allows the arms of the targeting constructs to consist entirely of intron sequences. In targeting the 5′ end of a gene in the pig, a synthetic Kozak-ATG consensus sequence was used to initiate translation as efficiently as does the endogenous translation start site. Neomycin-resistant targeted α1,3-GT colonies were detected at similar frequencies after transfection with either pGTIFneo or pGTKneo and selection with 600 μg/ml G418.

The cell lines used here were isolated from fetuses that were not sired from the boar whose DNA was used as template for generating the targeting constructs. Therefore, there was no chance that the cells possessed an allele isogenic to the constructs. The level of isogenicity and the extent of single base changes introduced by long-range PCR were not determined in this study. Dai et al. [10] found no differences between cell lines transfected with isogenic and nonisogenic DNA in the pig. In the present study, a similar rate of gene targeting was obtained with cell lines from two different fetuses. How these factors affect the rate of homologous recombination in the pig is unknown. As in humans [27], isogenic DNA is not an absolute requirement for gene targeting in pigs.

Unlike embryonic stem cells, somatic cells progress through a finite number of population doublings before becoming senescent, which places severe constraints on their ability to proliferate sufficiently to accommodate one round of gene targeting and cloning. An estimated 30–35 cell doublings are required for one round of gene targeting [28]. We optimized the conditions to culture, select, and identify the targeted clones, allowing successful expansion of the cells that carried the targeted α1,3-GT allele.

Targeted clones were efficiently detected by a preliminary PCR assay using specific primers that flanked the short 1.6-kb 5′ arm and the neomycin resistance gene. The forward primer was homologous to sequence outside the 5′ arm of the construct, and the reverse primer was anchored to homologous sequence within the 3′ arm. Using these flanking primers, we did not get the large number of false positives detected with targeting primers in which one primer is specific to sequence outside the construct and the other is specific to neomycin resistance gene sequence (data not shown) [9]. Because both the targeted mutant and nontargeted alleles had the same specific homologous binding sites for the preliminary PCR primers, both targeted and endogenous alleles were screened in the same reaction, making it easier to identify possible targets and mixed col-

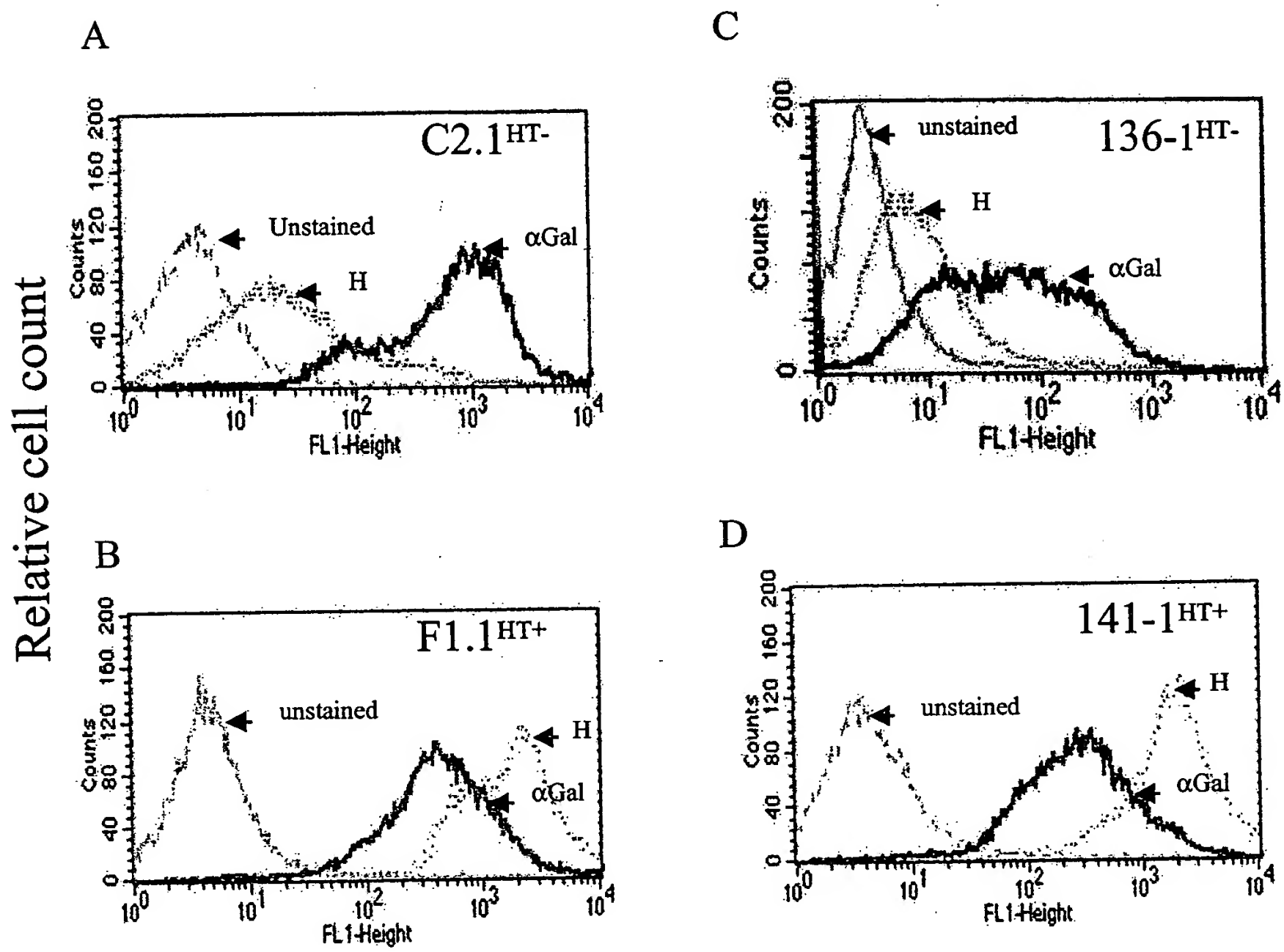


FIG. 6. Flow cytometric analysis of pig fibroblasts. The *x*-axis shows the fluorescence intensity, and the *y*-axis shows the relative cell count for unstained cells, H and Galα1,3-Gal antigen expression (arrowheads). A) Expression profile for the parental fibroblast cell line C2.1$^{HT-}$ from which piglets 136-1$^{HT-}$, 136-3$^{HT-}$, and 136-4$^{HT-}$ were derived. B) Expression profile for the parental fibroblast cell line F1.1$^{HT+}$ from which piglets 141-1$^{HT+}$ and 141-2$^{HT-}$ were derived. C) Expression profile for piglet 136-1$^{HT-}$, which was positive for Galα1,3-Gal but not H antigen. D) Expression profile for piglet 141-1$^{HT+}$, which was positive for Galα1,3-Gal and H antigens.

onies. All but one of the clones indicated as targeted by the preliminary PCR analysis were confirmed by long-range PCR analysis. In addition, results of the long-range PCR analysis were corroborated by Southern blotting, which confirmed the presence of a mutated α1,3-GT allele. The unique *Asc*I site placed after the neo selection cassette was used to cut the targeted fragment amplified by long-range PCR using primers that flanked the whole targeting construct so that it could be distinguished from the endogenous band by agarose gel electrophoresis. Long-range PCR permitted the detection of recombination events at both ends of the construct to confirm replacement of the WT with the disrupted allele.

The efficiency of homologous recombination found in this study ranged from 0.9% to 8.7%, as determined by long-range PCR analysis, and is comparable to that obtained in the two previous reports of α1,3-GT gene knockout pigs [9, 10]. Plating of the transfected cells in 100-mm tissue culture plates and selecting clones by the cloning ring method appears to isolate a higher percentage of targeted clones. Here, each colony identified as targeted by long-range PCR was isolated from a different plate and was assumed to represent an independent targeting event. However, isolating large numbers of clones by the cloning ring method is very tedious and time-consuming. The process can be scaled up to screen larger numbers of G418-resistant clones by selection in 96-well tissue culture plates. However, we observed a drop in targeting efficiency using this method that could be related to differences in plating densities. The targeting frequencies obtained for cell lines isolated from the two fetuses were similar when plated in the 96-well plates and when plated in the 100-mm plates. Although a slightly higher percentage of targeted clones was obtained with the pGTKneo construct, there was no significant difference from the percentage obtained with the pGTIFneo construct.

To date, we have generated six healthy α1,3-GT gene knockout male piglets, four of which express the HT antigen on their cells. Except for one apparently normal piglet that died soon after birth, the remaining seven piglets de-

rived from this work are healthy (including the HT-expressing nontargeted control littermate). Pregnancies were established with embryos reconstructed from all four targeted cell lines used for cloning.

This is the first study to show that Southern blot analysis can be performed to confirm a knockout in somatic pig cells before they are used for nuclear transfer. The two targeting constructs designed and used here were effective in replacing a WT allele at frequencies comparable to those previously reported for the α1,3-GT locus. A novel preliminary PCR screening strategy was developed and was stringent enough to avoid large numbers of false positives. A unique long-range PCR screening strategy also was developed and used to confirm the presence of the mutated allele by gene targeting. The long-range PCR screening strategy was equivalent in stringency to Southern blotting, as indicated by the similarities in numbers of targeted clones detected with both assays. In targeting the 5′ end of a gene in the pig, a synthetic Kozak-ATG consensus sequence was used to initiate translation as efficiently as does the endogenous translation start site. Thus, it is not necessary to clone the selection marker gene in frame with the endogenous ATG initiation codon. A consideration with this strategy is to retain the optimum distance of the Kozak sequence 5′ to the ATG within the untranslated region. We have produced healthy α1,3-GT gene knockout HT-transgenic male piglets that can be used to generate a α1,3-GT null phenotype. In future studies, the generation of homozygous α1,3-GT knockout pigs with the HT-transgenic background will be unique and may be more effective than the null phenotype alone in prolonging xenograft survival.

## ACKNOWLEDGMENTS

We gratefully acknowledge Naomi K. Bell and Jennifer Wynn for technical assistance.

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0041-1337/03/7606-900/0
TRANSPLANTATION


Vol. 76, 900–902, No. 6, September 27, 2003
*Printed in U.S.A.*

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RAPID COMMUNICATIONS

# HETEROZYGOUS DISRUPTION OF THE α1,3-GALACTOSYLTRANSFERASE GENE IN CATTLE

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***Background.*** **Animal cloning techniques have enabled gene disruption in several species. Here, we report the first successful disruption of the α1,3-galactosyltransferase (*α1,3-GT*) gene in cattle.**

***Methods.*** **The *α1,3-GT* gene of the Japanese Black cow (JBC) was used to construct pGT-6, a targeting vector for the bovine *α1,3-GT* gene, and pGT-6 was introduced into the fetal fibroblast cell line JBC906 by the lipofection method. Four polymerase chain reaction (PCR)-positive colonies were obtained from 797 G418-resistant colonies, and Southern blot analysis revealed successful homologous recombination at the *α1,3-GT* locus in one of the four colonies. Nuclear transfer was performed, and the four embryos were transferred to a heifer.**

***Results.*** **To establish fetal fibroblasts that were heterozygously disrupted at the *α1,3-GT* locus, one of the fetuses was recovered at 5 weeks of pregnancy, and PCR and Southern blot analysis of the fetal fibroblasts established from it showed definite homologous recombination of the *α1,3-GT* gene.**

***Conclusions.*** **Heterozygous knockout of the *α1,3-GT* gene was performed in JBC, and production of a homozygous *α1,3-GT* knockout JBC by a second round of targeting 906htGT is currently in progress. The technique described here can be applied to disruption of other genes in cattle.**

Targeted disruption of a particular gene used to be possible only in mice. However, several recent studies have reported successful gene disruption in sheep and pigs by animal cloning methods (*1–3*). Gene disruption in large animals is considered to be useful in medicine and in agriculture (*4*), and a major effort has been made in the medical field to disrupt the α1,3-galactosyltransferase (*α1,3-GT*) gene, which is the major barrier to xenotransplantation.

Cattle are potentially useful as donors for xenotransplantation, and although gene disruption is essential for achieving this goal, there have been no reports of gene disruption in bovine breeds. In this article, we report the establishment of heterozygous *α1,3-GT* knockout fetal fibroblast cells isolated from a fetal cloned Japanese Black cow (JBC).

## MATERIALS AND METHODS

### *Targeting Construct*

Because differences in sequence between the JBC and a commercially available bovine genomic DNA library might have affected the efficiency of homologous recombination, we created a JBC genomic DNA library, obtained an *α1,3-GT* gene, and used it to construct a promoterless targeting vector, pGT-6, by inserting an *IRES* (internal ribosome entry site)-β-galactosidase (*LacZ*) plus neomycin-resistance gene (*Neo*) cassette (*IRES-LacZ+Neo*) into exon 9 (Fig. 1). The pGT-6 contained a 6.3-kb *Eco*RV fragment (long arm) and an 0.8-kb *Hind*III-*Bam*HI fragment (short arm) of the *α1,3-GT* gene.

### *Cells and Gene Transfection*

JBC906 is a fetal fibroblast cell line that was established from a fetal JBC. To transfect the targeting vector, $2\times10^4$ JBC906 cells/well in 2 mL of MEM-α (Gibco BRL, Rockville, MD) containing 10% fetal calf serum (Research Institute for Functional Peptides, Yamagata, Japan) were plated out in four sets of six-well culture plates, and 2 days later 2 μg/well of pGT-6 that had been linearized with *Xho*I was introduced with TransFast transfection reagent (Promega, Madison, WI). The transfection protocol was that recommended by the manufacturer. After 24 hr, 0.4 mg/mL G418 (Geneticin; Gibco BRL) was added to the culture medium, and the next day cells were collected and seeded into eight sets of 48-well culture plates. The culture medium was replaced every 4 days. At 12 to 14 days after transfection, the G418-resistant colonies were divided into two wells of a 48-well tissue-culture plate. One well was used for polymerase chain reaction (PCR), and the other well was used for Southern blot analysis and nuclear transfer.

### *PCR Analysis*

Cells were suspended in 50 μL of lysis buffer (10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.45% NP-40, 0.45% Triton-X100, and 60 μg/mL proteinase K) and then incubated at 55°C for 70 min and 100°C for 13 min. Lysates (5 μL/reaction) were directly subjected to PCR. Amplification was performed using an anti-Taq high kit (Toyobo, Tokyo, Japan) and a reaction volume of 25 μL. Cycling conditions were 1 cycle of 1 min at 94°C; 35 cycles of 1 min at 94°C, 1 min 30 s at 57°C, 2 min at 72°C; and 1 cycle of 10 min at 72°C. The

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Received 30 January 2003. Revision requested 24 April 2003. Accepted 15 May 2003.
DOI: 10.1097/01.TP.0000084300.29416.AD

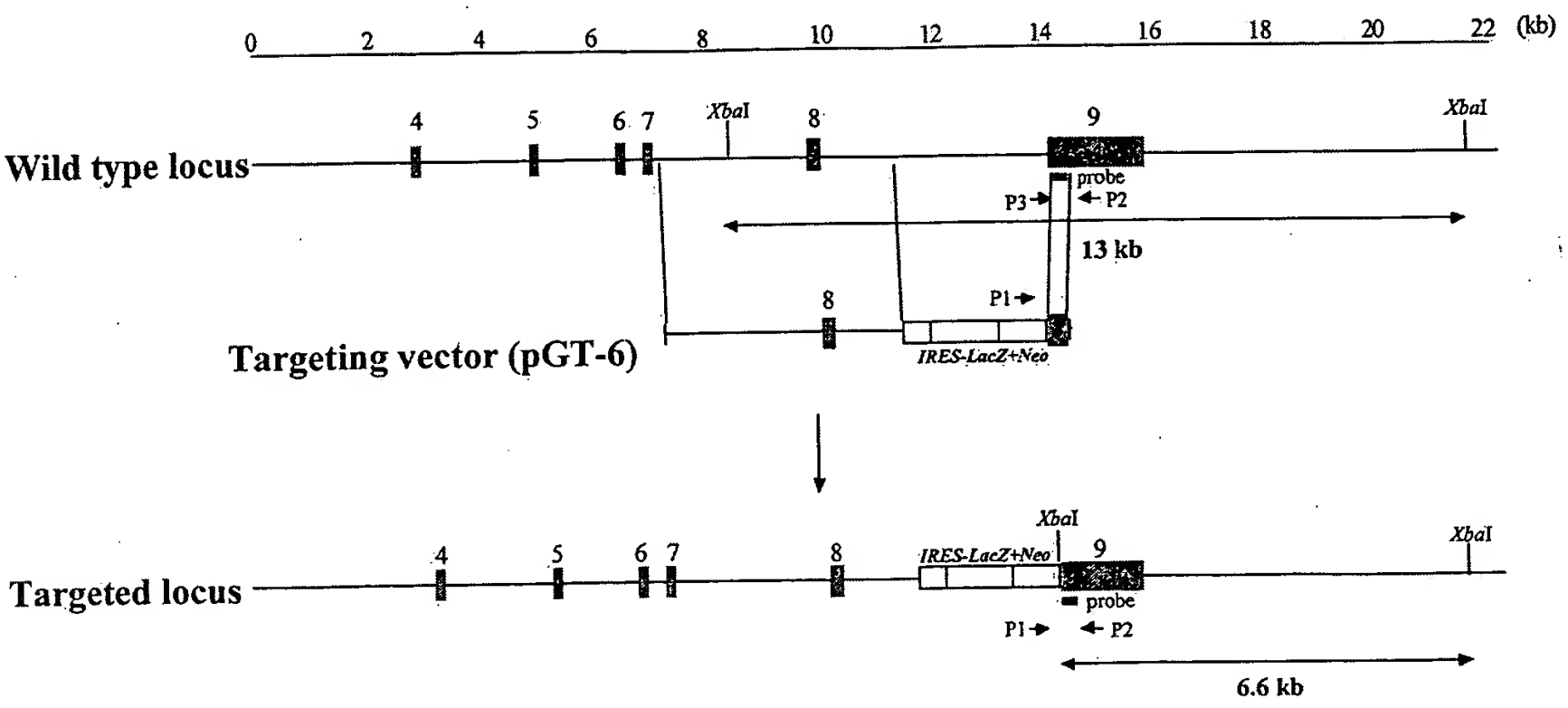


**FIGURE 1. Diagram of the bovine *α1,3-GT* locus and targeting vector. (*shaded boxes*) Exons; (*white boxes*) targeting cassette *IRES-LacZ+Neo*; (*P1*, *P2*, and *P3*) PCR primers used in this study (see Fig. 2A).**

primer locations are shown in Figure 1: P1, 5-ACCGCTATCAGGACATAGCGTTGG-3; P2, 5-CTGGAGACCACCCACACTGCCTGG-3; and P3, 5-GATTATCACATAGGCCTACCTGCGG-3. Products were analyzed by 1% agarose gel electrophoresis. A positive signal (P1–P2 product) was detected at 1.2 kb.

### *Southern Blot Analysis*

After digesting 15 μg of genomic DNA with *Xba*I and separation on 0.8% agarose gel, the DNA was transferred to a nylon membrane, and signals were detected with a DNA probe corresponding to the short arm of the pGT-6 vector. The signal for the wild type was detected at 13 kb, and the signal for the homologous recombinant was detected at 6.6 kb.

### *Nuclear Transfer and Establishment of the Fetal Fibroblast Cell Line*

Oocytes matured in vitro were enucleated 19 to 20 hr after the start of culture, and donor cells (3–28) were cultured at 38°C for 3 days in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) containing 10% FCS under an atmosphere of 5% carbon dioxide and 95% air. Enucleated oocytes were placed next to donor cells, and fusion was performed by application of a DC pulse of 25 V/150 μm for 10 μs, followed by a DC pulse of 100 V/mm for 60 μs. Activation was performed by exposure to Ca-ionophore (5 μM, 5 min) and cycloheximide (10 μg/mL). Fused cells were co-cultured at 39°C with bovine oviductal epithelial cells for 6 days in a humidified atmosphere of 5% carbon dioxide in air, and the embryos were transferred to the uterus of a recipient Holstein heifer. At day 38 of gestation, the heifer was killed and one fetus was recovered. Day 38 was chosen because our previous studies had indicated that fetal JBC fibroblasts recovered at this stage had the best qualities for cell culture assay and for use as donor cells for nuclear transfer. Our strategy was to use the fibroblasts for second-round targeting. Because pregnancy lasts approximately 10 months in cattle, it takes a long time to produce homozygotes by backcrossing. Second-round targeting of heterozygous gene-disrupted fibroblasts is a far cheaper and more rapid means of doing so.

The head and digestive tract of the fetus were removed, and the rest of the fetus was digested with 0.1% trypsin overnight at 37°C. The fibroblasts recovered were cultured for 5 days in DMEM containing 10% FCS and then stored in liquid nitrogen.

## RESULTS AND DISCUSSION

JBC have been extensively investigated in nuclear transfer studies in Japan (*5*) and have potential advantages as donors for xenotransplantation, one of which is that they are smaller than other bovine breeds. We assessed four different fetal fibroblast cell lines for efficiency of growth and transduction, and selected the JBC906 cell line for the experiments.

Six transfection experiments were performed, and 797 G418-resistant colonies were obtained (Table 1). Four of these 797 colonies were PCR-positive for *α1,3-GT* targeting (colonies 1–59, 3–28, 1–26, and 1–34)(Fig. 2A), and all of them were successfully expanded for Southern blot analysis. Southern blot analysis revealed disruption at the *α1,3-GT* gene in colony 3–28 alone (Fig. 2B). Colonies 1–26 and 1–34 contained an extra signal (Fig. 2B) around 6.6 kb, suggesting that both successful homologous recombination and random integration had occurred in the same cell and that they were derived from the same colony, and colony 1–59 contained only the endogenous *α1,3-GT* allele. We therefore used cells from colony 3–28 as the donor cells for nuclear transfer.

**TABLE 1. Summary of in vitro targeting**

| Experiment No. | G418-resistant colony | PCR-positive clone |
|---|---|---|
| 1 | 126 | 1 (1–59) |
| 2 | 82 | 0 |
| 3 | 116 | 1 (3–28) |
| 4 | 122 | 0 |
| 5 | 196 | 2 (1–26, 1–34) |
| 6 | 155 | 0 |
| Total | 797 | 4 |

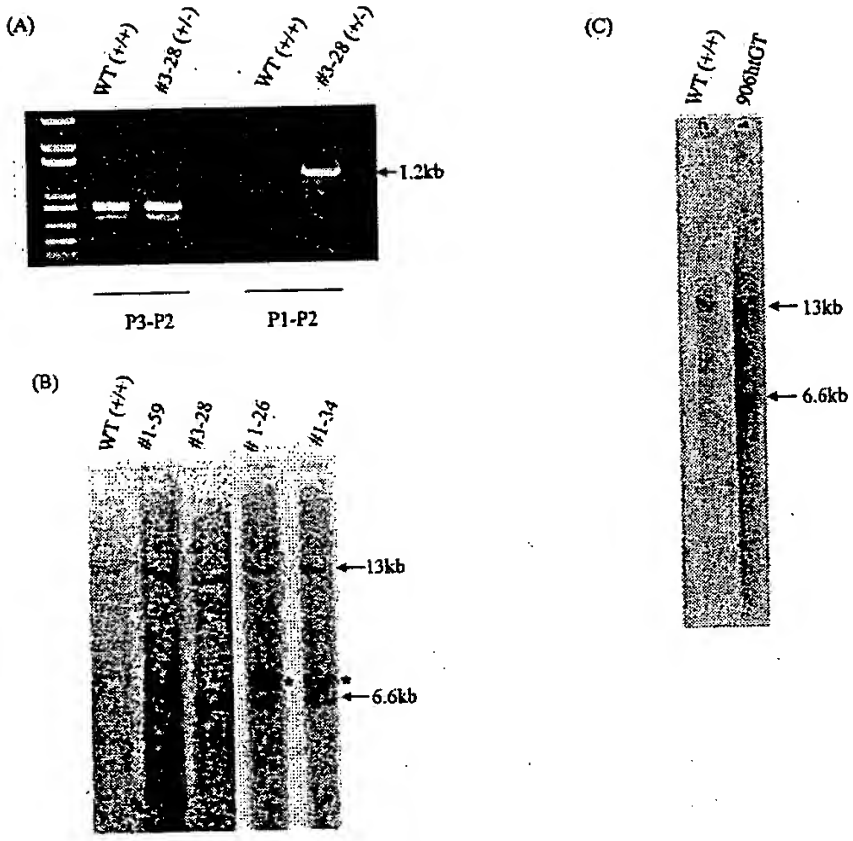


**FIGURE 2. (A) PCR results for G418-resistant colonies. WT, wild type. (B) Southern blot analysis of G418-resistant colonies. The wild-type allele produces a signal at 13 kb, and the homologous recombinant produces a signal at 6.6 kb. (C) Southern blot analysis of 906htGT.**

Nuclear transfer was performed as described earlier (see *Materials and Methods*). Forty cells were pulsed and fused, and 35 of them had cleaved to the 2- to 8-cell stage by day 3 (Table 2). By day 6, 24 cleaved embryos had developed to the compact morula stage, and four compact morulas were ultimately transferred to a recipient heifer. Only one fetus was recovered at 5 weeks of pregnancy, and macroscopic examination showed no apparent abnormalities. We killed the fetus at this stage to establish heterozygously gene-disrupted fetal fibroblasts for use in second-round targeting. Our strategy is faster and more economical than backcrossing cattle to produce homozygous disruptions, and it has considerable future potential for producing homozygous disruption of any genes in cattle.

**TABLE 2. Summary of nuclear transfer**

| No. of pulsed embryos | No. cleaved to 2–8 cells (day 3) | No. that developed to a compacted morula (day 6) |
|---|---|---|
| 40 | 35 (87.5%) | 24 (60.0%) |

The fetus had a crown-rump length of 19 mm and weighed 1 g. Both values were within the normal range. Fetal fibroblast cell line 906htGT was established from the fetus, and Southern blot analysis of the 906htGT cells showed definite disruption at the *α1,3-GT* gene (Fig. 2C).

We expect that *α1,3-GT* knockout JBC will serve as an alternative to miniature swine as a source of xenogeneic donors. The small size of adult JBC enables donor kidneys to be obtained from them, and possible size mismatches can be overcome by surgical reduction. Donation of hearts and lungs by fetuses offers potential advantages. Tissues and cells can be donated by an adult JBC, regardless of size, and isolation of bovine pancreatic islets is much easier than isolation of porcine islets (*6*).

## CONCLUSION

We are currently in the process of producing homozygous *α1,3-GT* knockout JBC by a second round of 906htGT targeting. This technique offers a new means of producing genetically engineered cattle.

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# $\alpha$1,3-Galactosyltransferase Gene-Knockout Miniature Swine Produce Natural Cytotoxic Anti-Gal Antibodies

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**Background.** The expression of galactose$\alpha$1,3galactose (Gal) in pigs has proved a barrier to xenotransplantation. Miniature swine lacking Gal ($Gal^{-/-}$ pigs) have been produced by nuclear transfer/embryo transfer.

**Methods.** The tissues of five $Gal^{-/-}$ pigs of SLA dd haplotype ($SLA^{dd}$) were tested for the presence of Gal epitopes by staining with the *Griffonia simplicifolia* IB4 lectin. Their sera were tested by flow cytometry for binding of IgM and IgG to peripheral blood mononuclear cells (PBMC) from wild-type ($Gal^{+/+}$) SLA-matched pigs; serum cytotoxicity was also assessed. The cellular responses of PBMC from $Gal^{-/-}$ swine toward $Gal^{+/+}$ SLA-matched PBMC were tested by mixed leukocyte reaction and cell-mediated lympholysis assays.

**Results.** None of the tissues tested showed Gal expression. Sera from all five $Gal^{-/-}$ pigs manifested IgM binding to $Gal^{+/+}$ pig PBMC, and sera from three showed IgG binding. In all five cases, cytotoxicity to $Gal^{+/+}$ cells could be demonstrated, which was lost after treatment of the sera with dithiothreitol, indicating IgM antibody-mediated cytotoxicity. PBMC from $Gal^{-/-}$ swine had no proliferative or cytolytic T-cell response toward $Gal^{+/+}$ SLA-matched PBMC.

**Conclusions.** $Gal^{-/-}$ pigs do not express Gal epitopes and develop anti-Gal antibodies that are cytotoxic to $Gal^{+/+}$ pig cells. The absence of an in vitro cellular immune response between $Gal^{-/-}$ and $Gal^{+/+}$ pigs is related to their identical SLA haplotype and indicates the absence of immunogenicity of Gal in T-cell responses. The model of $Gal^{+/+}$ organ transplantation into a $Gal^{-/-}$ SLA-matched recipient would be a valuable large animal model in the study of accommodation or B-cell tolerance.

(*Transplantation* 2004;78: 15–20)

A new era of xenotransplantation research has commenced with the availability of pigs in which the $\alpha$1,3-galactosyltransferase gene has been knocked out and which therefore do not express galactose$\alpha$1,3galactose (Gal) ($Gal^{-/-}$ pigs) (*1*). The presence of natural antibodies (Ab) to the Gal epitope has been a major barrier to successful xenotransplantation in pig-to-nonhuman primate models. Many groups have attempted to overcome hyperacute rejection and acute humoral xenograft rejection mediated by anti-Gal Ab using different strategies to manipulate production and/or function of these Ab. The availability of $Gal^{-/-}$ pigs enables the investigation of pig-to-baboon organ transplantation in the absence of anti-Gal Ab-mediated rejection; it also offers a large animal model to study other immunologic phenomena that could not be investigated in large animals previously, such as accommodation and B-cell tolerance.

F.J.M.F.D. is a recipient of grants from the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences, the Prof. Michaël van Vloten Fund, and the Netherland-America Foundation. Y.-L.T. is a recipient of a grant from the National Cheng Kung University Hospital, Taiwan. D.H.S. and D.K.C.C. are consultants to Immerge BioTherapeuthics. This work was supported in part by National Institutes of Health Program Project 1PO1 A145897 and by a Sponsored Research Agreement between the Massachusetts General Hospital and Immerge BioTherapeutics, Inc.

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Received March 5, 2004. Accepted April 6, 2004.



ISSN 0041-1337/04/7801-15

DOI: 10.1097/01.TP.0000130487.68051.EB

## METHODS

### Animals

Five $Gal^{-/-}$ miniature swine, generated by nuclear transfer/embryo transfer from modified fibroblasts from Massachusetts General Hospital (MGH) MHC-inbred miniature swine of swine leukocyte antigen $(SLA)^{dd}$ haplotype, served as sources of tissues, sera, and peripheral blood mononuclear cells (PBMC). Swine ranged in age from 2.8 to 4.2 months. Homozygosity for $\alpha$1,3-galactosyltransferase gene-knockout was confirmed by genomic polymerase chain reaction and genomic Southern blots (*1a*). Tissues, sera, and PBMC were also obtained from 15 wild-type ($Gal^{+/+}$) MGH miniature swine matched for age to the $Gal^{-/-}$ pigs.

### Preparation and Staining of Tissues

Biopsy specimens of all major organs and tissues (Table 1) were obtained from freshly killed $Gal^{-/-}$ and $Gal^{+/+}$ pigs. Specimens were fixed in 10% buffered formalin and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin.

To detect Gal, the immunoperoxidase technique with the biotinylated lectin *Griffonia simplicifolia* (IB4) was used (*2,3*). Four-micron sections were treated in BorgDeclocker solution (Biocare Medical, Walnut Creek, CA) for 3 min at 120°C in a pressure cooker (Biocare Medical) and incubated with biotinylated IB4 lectin (Vector Laboratories, Burlingame, CA) for 30 min at predetermined optimal dilution. After washing with phosphate-buffered saline, the sections were incubated with avidin/biotin peroxidase complex (Vectastain Elite ABC Kit; Vector) for 30 min, visualized by using a 3,3'-diaminobenzidine substrate kit (Vector), and counterstained with hematoxylin.

**TABLE 1.** Staining of tissues from $Gal^{-/-}$ and $Gal^{+/+}$ miniature swine with IB4 lectin

| Tissue | $Gal^{-/-}$ pig | $Gal^{+/+}$ pig |
|---|---|---|
| Heart | Negative | CP, BV |
| Kidney | Negative | CP and capillaries in glomerular loops, BV, some tubular epithelial cells |
| Lung | Negative | BV, MΦ, alveolar epithelial cells, bronchial epithelial cells, CP |
| Pancreas | Negative | CP, BV, centro-acinar cells |
| Liver | Negative | BV, Kupffer cells, some hepatocytes[a] |
| Spleen | Negative | BV, lymphoid cells, MΦ, DC, stromal cells |
| Thymus | Negative | CP, BV, thymocytes, MΦ, DC |
| Intestine-small | Negative | CP, BV, cells in lamina propria, muscle cells in muscle layer |
| Intestine-large | Negative | CP, BV, cells in lamina propria, muscle cells in muscle layer |
| Ureter | Negative | CP, BV, muscle cells in muscle layer, epithelial cells in basal layer |
| Stomach | Negative | CP, BV, cells in lamina propria, muscle cells in muscle layer |
| Bone marrow | Negative | Almost all cells |
| Aorta | Negative | CP, BV, endothelial cells |
| Salivary gland | Negative | CP, BV, myoepithelial cells |
| Bladder | Negative | CP, BV, muscle cells in muscle layer, epithelial cells |
| Lymph node | Negative | Lymphoid cells, MΦ, DC, BV, CP |
| Tonsil | Negative | Lymphoid cells, MΦ, DC, epithelial cells, BV, CP |
| Ear | Negative | CP, epithelial cells in basal layer, BV |
| Skin | Negative | CP, epithelial cells in basal layer, BV |
| Ovary | Negative | CP, BV, germinal epithelial cells |

[a] Hepatocytes always have some background staining.
CP, capillaries; BV, blood vessels; MΦ, macrophage; DC, dendritic cells.

### Isolation of PBMC from Blood

Heparinized whole blood was diluted with Hanks' balanced salt solution (Gibco, Grand Island, NY) followed by density gradient centrifugation (30 min, 1400*g*) using lymphocyte separation medium (ICN Biomedicals, Aurora, OH). The remaining red blood cells in harvested buffy coats were lysed using ACK lysing buffer (BioWhittaker, Walkersville, MD). Cells were stored in mixed leukocyte reaction (MLR) media (RPMI 1640 without L-glutamine [Gibco]) supplemented with 6% fetal pig serum (Sigma Chemical, St. Louis, MO), 100 U/mL penicillin, 135 μg/mL streptomycin (Gibco), 50 μg/mL gentamicin (Gibco), 10 mM HEPES (Fisher Scientific, Pittsburgh, PA), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (BioWhittaker), 1% nonessential amino acids mixture (100×) (BioWhittaker), and $5\times10^{-5}$ M β2 mercaptoethanol (Sigma) until usage. These PBMC were used in flow cytometry, cytotoxicity, MLR, and cell-mediated lympholysis (CML) assays.

### Enzyme-Linked Immunoadsorbent Assay for Natural Anti-Gal Antibodies

This procedure followed the previously described method (4). Briefly, 96-well plates coated with 5 μg/mL bovine serum albumin (BSA)-Gal (Alberta Research Council, Edmonton, Alberta, Canada) and blocked with 1% BSA in PBS-Tween were loaded with graded doses of pig serum starting at 2% and diluted fivefold afterward, followed by incubation with horseradish peroxidase-conjugated rabbit Ab specific for pig IgG or IgM. Color development was achieved by ABTS and stopped by 2N sulfuric acid after 13-min incubation in the dark. Plates were read using a Thermomax plate reader (Molecular Devices, Menlo Park, CA).

### Flow Cytometry for the Detection of Antibodies to $Gal^{+/+}$ MHC-matched PBMC in Serum of $Gal^{-/-}$ Pigs

Sera of $Gal^{-/-}$ pigs were heat-inactivated for 30 min at 56° C. PBMC ($10^6$) from $Gal^{+/+}$ $SLA^{dd}$ miniature swine in 100 μL of FACS buffer (Hanks' balanced salt solution containing $Ca^{2+}$ and $Mg^{2+}$/0.1% BSA/0.1% $NaN_3$) were incubated with 10 μL of pig immunoglobulin (50 μg) (blockade of nonspecific binding through, eg, Fc receptors). Then, 10 μL of heat-inactivated $Gal^{-/-}$ serum was added to the designated test tubes. The positive control consisted of heat-inactivated serum from a $SLA^{ac}$ pig that was immunized with $SLA^{dd}$ cells, and the negative control consisted of heat-inactivated normal pig serum. After 30 min of incubation, optimal concentrations of the secondary Ab (polyclonal fluorescein isothiocyanate-conjugated goat anti-swine IgG and IgM) were added. Additional negative controls included $Gal^{+/+}$ $SLA^{dd}$ PBMC alone and PBMC incubated with the secondary Ab only. Data were acquired using a Becton Dickinson FACScan (San Jose, CA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME).

### Determination of Cytotoxic Antibodies to $Gal^{+/+}$ PBMC in Serum of $Gal^{-/-}$ Pigs

Target cell suspensions ($Gal^{+/+}$ $SLA^{dd}$ PBMC) were diluted to $5\times10^6$ cells/mL in medium 199 (Cellgro, Herndon, VA) supplemented with 2% fetal calf serum (culture media).

Serum samples were serially diluted in culture media from 1:2 to 1:2048. The effect of dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO) on cytotoxicity was tested by adding DTT to the serum at 1:20, 1:40, and 1:80 dilutions. In 96-well U-bottom plates (Costar, Cambridge, MA), 25 $\mu$L of the appropriate target cell suspension ($Gal^{+/+}$ $SLA^{dd}$ PBMC) was incubated with 25 $\mu$L of diluted serum or controls, for 15 min at 37°C. A second incubation was performed with 25 $\mu$L of diluted rabbit complement, at a predetermined working dilution in culture media at 37°C for 30 min. After washing, dead cells were identified by trypan blue dye exclusion, until a total of 100 cells were counted. The number of dead cells was equivalent to the percentage of dead cells present in each well.

### In Vitro Assays for the Determination of T-Cell Responses from $Gal^{-/-}$ Pigs Toward $Gal^{+/+}$ Pigs

In MLR and CML assays, PBMC from $Gal^{-/-}$ pigs were used as responders (MLR) or effectors (CML), with PBMC from three different $Gal^{+/+}$ $SLA^{dd}$ MHC-matched pigs, third-party $Gal^{+/+}$ $SLA^{cc}$, or PBMC from the responder pig itself, as stimulators. As controls, PBMC from a $Gal^{+/+}$ $SLA^{dd}$ pig were used as responders to the same $Gal^{+/+}$ $SLA^{dd}$ and $Gal^{-/-}$ $SLA^{dd}$ stimulators.

### Mixed Leukocyte Reaction Assay

MLR co-cultures were performed as described previously (5). Briefly, 100 $\mu$L ($4\times10^5$ cells) of responder PBMC were incubated in a flat-bottomed 96-well plate (Costar) with 100 $\mu$L ($4\times10^5$ cells) of irradiated (2500 cGy) PBMC for 5 days in humidified air at 37° C, 5% $CO_2$ (in triplicate). A total of 1 $\mu$Ci of [$^3$H]thymidine was added to each well, followed by an additional 6-h incubation, and cells were harvested using a TomTek harvester (Perkin Elmer Wallac, Gaithersburg, MD). [$^3$H]thymidine incorporation was measured as counts per minute (cpm) using the Microbeta liquid-scintillation system (Perkin Elmer Wallac, Norwalk, CT).

### Cell-Mediated Lympholysis Assay

Media for CML co-cultures were similar to MLR media, except for the use of fetal bovine serum instead of fetal pig serum. Media used for the effector phase of CML assays consisted of Basal Medium Eagle (GIBCO BRL) supplemented with 6% serum replacement medium (Sigma-Aldrich). CML assays were performed as described previously (*6,7*). Briefly, cultures of lymphocytes containing $4\times10^6$ responder and $4\times10^6$ irradiated (2500 cGy) stimulator PBMCs in 2 mL of medium were incubated for 6 days at 37°C in 7% $CO_2$ and 100% humidity. Bulk cultures were harvested, and effector cells were tested on a panel of $^{51}$Cr-labeled lymphoblasts generated by phytohemagglutinin stimulation. The cytolytic tests were run by incubating standard $5\times10^3$ target cells with effector cells at serially diluted effector/target ratios (100:1, 50:1, 25:1, and 12.5:1 for 5.5 h). The supernatants were harvested, and $^{51}$Cr release was determined on a gamma counter. Baseline levels were measured as the rate of spontaneous release of $^{51}$Cr from $5\times10^3$ targets. Maximal lysis was determined as the release of $^{51}$Cr from $5\times10^3$ targets after incubation with 5% NP-40 (United States Biochemical, Cleveland, OH). The results were expressed as percentage specific lysis (PSL): % specific lysis = [experimental release (cpm) − spontaneous release (cpm) / maximum release (cpm) − spontaneous release (cpm)] × 100.

## RESULTS

### Tissues from $Gal^{-/-}$ Miniature Swine Do Not Express Gal Epitopes

None of the tissues stained with IB4 showed expression of Gal (Table 1 and Fig. 1), in contrast to tissues from $Gal^{+/+}$ pigs that have been reported previously (*2,8,9*).

### $Gal^{-/-}$ Miniature Swine Produce Anti-Gal Antibodies That Are Cytotoxic to $Gal^{+/+}$ MHC-Matched PBMC

All five $Gal^{-/-}$ pigs had IgM that bound to $Gal^{+/+}$ pig PBMC (Fig. 2A), and three of the five also had IgG (Fig. 2B). These flow cytometry results were confirmed by ELISA. All five sera were cytotoxic to $Gal^{+/+}$ PBMC (Fig. 3); the cytotoxicity was lost after treatment of the sera with 1:20 dithiothreitol (Sigma-Aldrich) (Fig. 3B), indicating that cytotoxicity was mediated by IgM-class Ab. There appeared no correlation between either the presence of anti-Gal IgG or the extent of cytotoxicity with the age of the $Gal^{-/-}$ pigs (which ranged from 2.8–4.2 mo) or the duration of stay at our facility (which was 22–62 days).

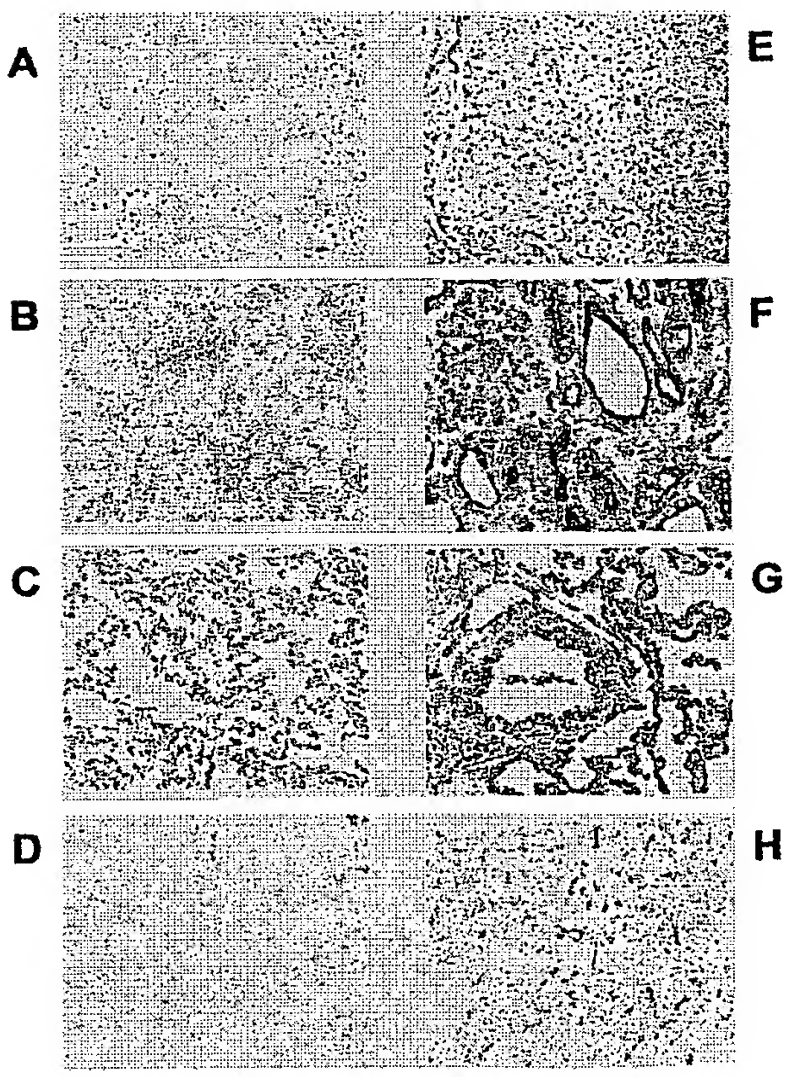


**FIGURE 1.** Histologic sections of heart (A and E), kidney (B and F), lung (C and G), and pancreas (D and H) from $Gal^{-/-}$ (A–D) and $Gal^{+/+}$ (E–H) SLA-matched miniature swine, stained with IB4. No expression of Gal was detected on the $Gal^{-/-}$ tissues.

### $Gal^{-/-}$ Pigs Do Not Generate a Cellular Response to $Gal^{+/+}$ SLA-Matched Miniature Swine

PBMC from $Gal^{-/-}$ miniature swine (of $SLA^{dd}$ haplotype) showed no proliferative response (on MLR) (Fig. 4) and no lytic response (on CML) (Fig. 5) toward SLA-matched $Gal^{+/+}$ PBMC. The reverse was also true: $Gal^{+/+}$ pigs mounted no cellular response to $Gal^{-/-}$ MHC-matched cells (not shown). Both $Gal^{-/-}$ pigs and SLA-matched $Gal^{+/+}$ pigs demonstrated good proliferative and cytolytic responses to third-party $Gal^{+/+}$ $SLA^{cc}$ stimulators (Figs. 4 and 5).

## DISCUSSION

The $Gal^{-/-}$ miniature swine generated by nuclear transfer/embryo transfer did not express Gal on any of the organs or tissues tested. The absence of the expression of Gal enabled them to produce anti-Gal Ab, probably in response to colonization of their gastrointestinal tracts by microorganisms expressing surface Gal antigens (*10*). Similar observations have been made in $Gal^{-/-}$ mice (*11*). The pig Ab were predominantly IgM, although some IgG was also identified; the IgM was cytotoxic to SLA-matched $Gal^{+/+}$ pig cells. Although the range of age of the pigs was small and the duration of their stays at our facility was relatively short, we did not find a correlation between either pig age or length of exposure to our institutional environment with the level of anti-Gal IgG or the extent of serum cytotoxicity; however, a correlation may have been found if the pigs had been maintained to an older age or if the duration of stay at our facility had been longer. As the $Gal^{-/-}$ and $Gal^{+/+}$ miniature swine were of identical SLA haplotype, neither generated a cellular immune response to the other's cells in vitro.

In our studies, we could not demonstrate in vitro immunogenicity of the Gal epitope in T-cell responses. However, we did not study natural killer cell or macrophage reactivity. Despite the SLA identity in the pigs we studied, the ability of the $Gal^{-/-}$ pigs to make anti-Gal Ab should enable them to reject a transplanted $Gal^{+/+}$ pig organ by an Ab-mediated mechanism, either hyperacutely or in a delayed manner. Future studies are needed to determine whether natural anti-Gal Ab in the $Gal^{-/-}$ pig are able to cause hyperacute rejection or whether some form of sensitization is required. The model of $Gal^{+/+}$ organ transplantation into a $Gal^{-/-}$ SLA-matched recipient could be an ideal large animal model for the study of accommodation or B-cell tolerance.

## ACKNOWLEDGMENTS

*The authors thank Leo H. Buhler, M.D., and Kenji Kuwaki, M.D., for their comments on this manuscript.*

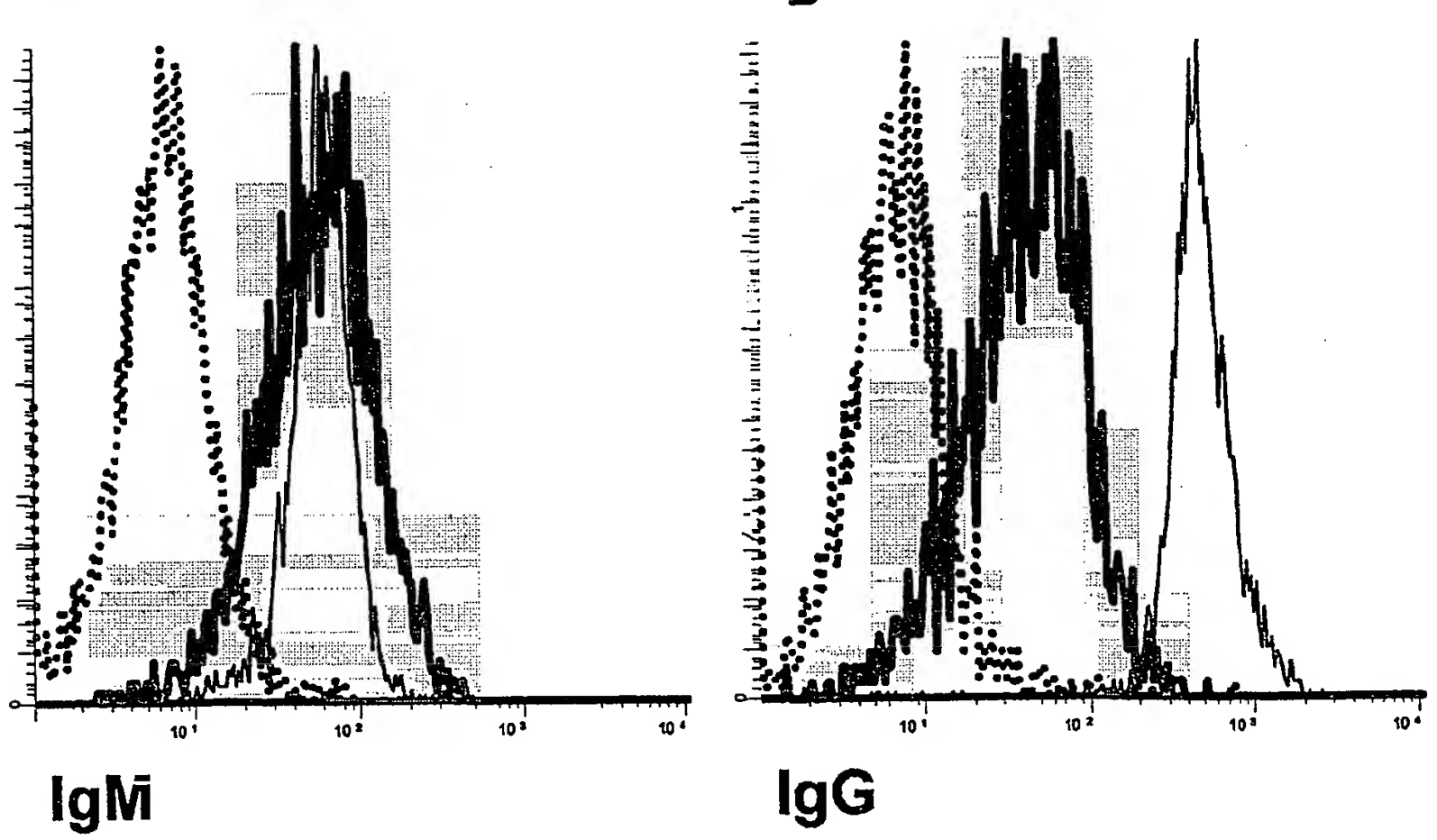


**FIGURE 2.** Flow cytometry demonstrating binding of IgM (A) and IgG (B) from serum of a $Gal^{-/-}$ miniature swine to PBMC from a $Gal^{+/+}$ SLA-matched miniature swine. Normal pig serum (negative control; *dashed line*); anti-DD pig serum (positive control; *thin line*); serum from a $Gal^{-/-}$ miniature swine (experimental sample; *bold line*). This particular $Gal^{-/-}$ pig serum contained both anti-Gal IgM (with similar binding to the positive control) and IgG (with less binding than the positive control).

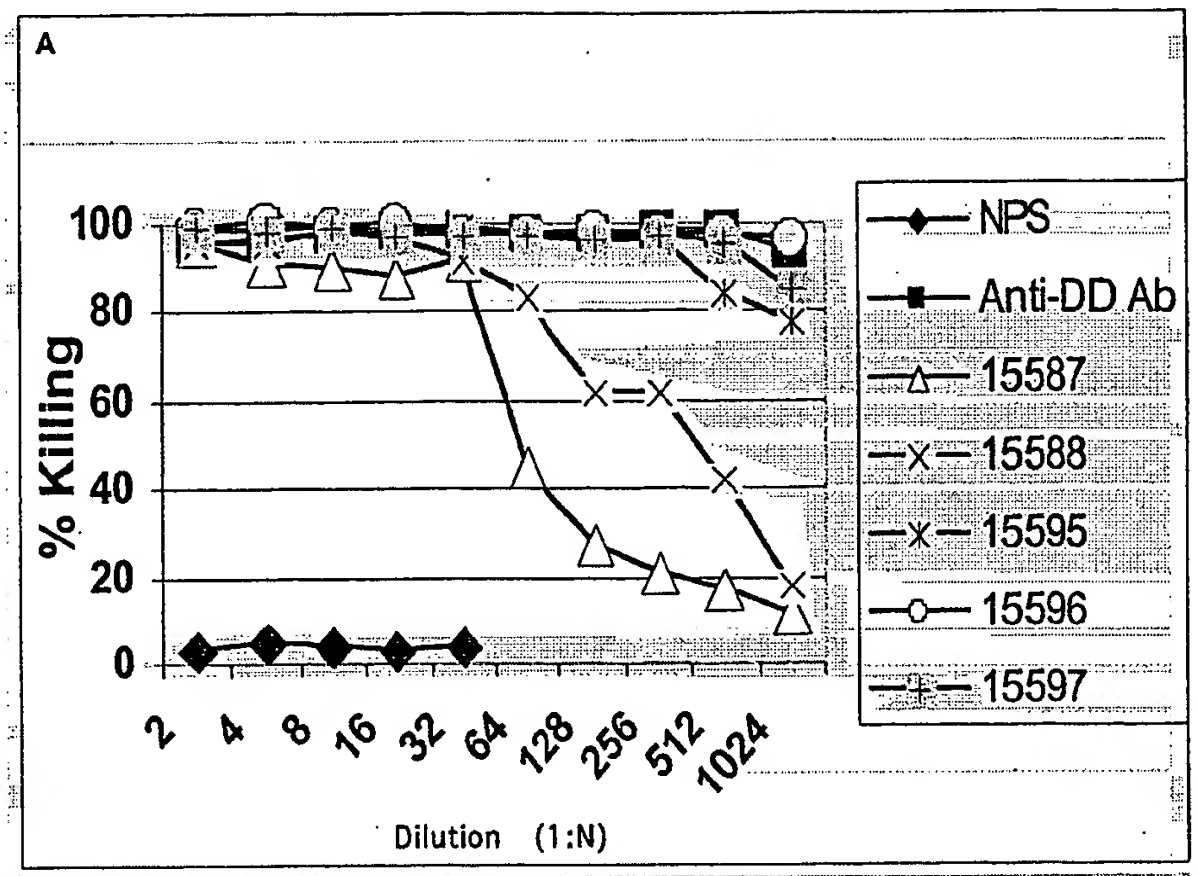


**FIGURE 3.** Results of cytotoxicity assay of sera from five $Gal^{-/-}$ miniature swine (numbered 15587, 15588, 15595, 15596, and 15597) toward PBMC from a $Gal^{+/+}$ SLA-matched miniature swine. All $Gal^{-/-}$ sera were cytotoxic to $Gal^{+/+}$ PBMC despite their SLA identity (A). Cytotoxicity was lost after treatment of the sera with DTT at a 1:20 dilution. Shown here is the serum of $Gal^{-/-}$ pig 15595; dilutions of DTT at 1:80 and 1:40 demonstrated a titratable decrease in cytotoxicity but an incomplete effect (B). NPS, normal pig serum.

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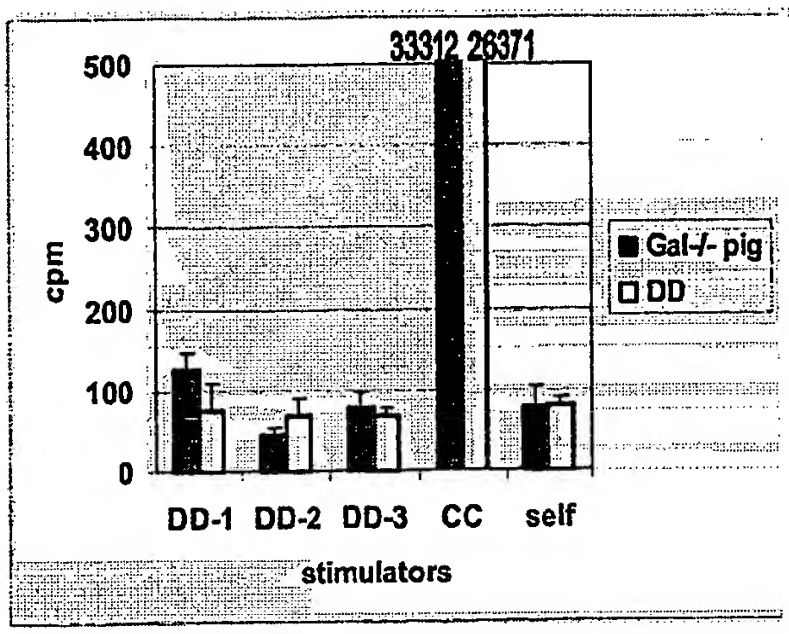


**FIGURE 4.** Representative results of MLR assay after co-culture of PBMC from a $Gal^{-/-}$ miniature swine of $SLA^{dd}$ haplotype with PBMC from three different $Gal^{+/+}$ SLA-matched swine (DD-1, DD-2, DD-3) and one SLA-mismatched $Gal^{+/+}$ swine (CC). The PBMC from the $Gal^{-/-}$ swine demonstrated a normal proliferative (allo) response toward PBMC of the $SLA^{cc}$ haplotype (CC) (cpm 33,312 in $Gal^{-/-}$ and 26,371 in $Gal^{+/+}$ swine, respectively, as indicated above the bars) but no response toward $Gal^{+/+}$ PBMC of $SLA^{dd}$ haplotype (similar to the self-response). In a $Gal^{+/+}$ swine of $SLA^{dd}$ haplotype, the MLR responses to the different stimulators were similar.

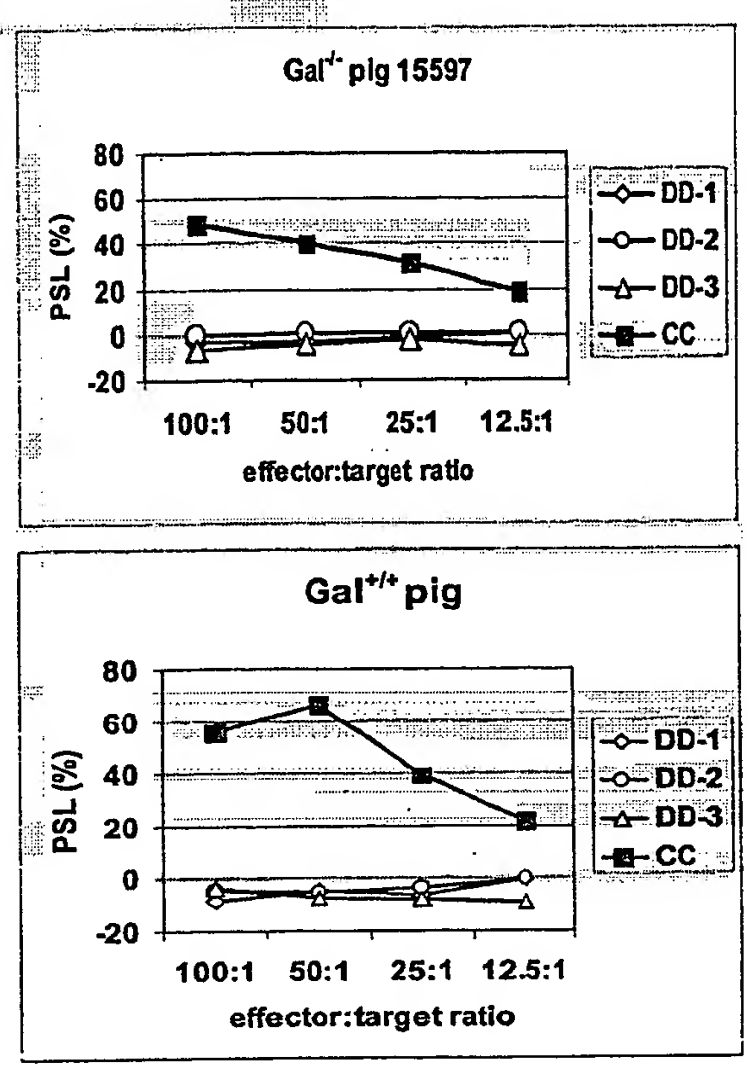


**FIGURE 5.** (A) Representative results of CML assay after co-culture of PBMC from a $Gal^{-/-}$ miniature swine of $SLA^{dd}$ haplotype with PBMC from three different $Gal^{+/+}$ SLA-matched swine (DD-1, DD-2, DD-3) and one SLA-mismatched $Gal^{+/+}$ swine (CC). The PBMC from the $Gal^{-/-}$ swine demonstrated a normal lytic (allo) response toward PBMC of the $SLA^{cc}$ haplotype (CC), but no response toward $Gal^{+/+}$ PBMC of $SLA^{dd}$ haplotype (similar to the self-response). (B) The CML responses of a $Gal^{-/-}$ swine of $SLA^{dd}$ haplotype are similar to those of a $Gal^{+/+}$ swine of $SLA^{dd}$ haplotype. PSL, percentage specific lysis.

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# Long-term experience with porcine aortic valve xenografts

*Between 1971 and 1975, glutaraldehyde-preserved porcine aortic valve xenografts were employed for isolated replacement of the mitral valve (MVR) in 243 patients, replacement of the aortic valve (AVR) in 167 patients, and double valve replacement (AVR and MVR) in 51 patients. Postoperatively, long-term anticoagulation was not routinely given. Operative mortality rates for AVR, MVR, and double valve groups were 7.8, 6.0, and 11.8 per cent, respectively; the majority of early postoperative deaths were associated with concomitant coronary artery disease. No death was attributable to xenograft dysfunction. Follow-up of all patients was obtained. The total duration of follow-up for the MVR group was 347 patient-years, for the AVR group 148 patient-years, and for double valve replacement 37 patient-years; maximum follow-up for these three groups was 4.4, 4.0, and 2.4 years, respectively. Actuarial analysis of postoperative survival rates at a common interval of 3 years showed 78 per cent for MVR patients, 91 per cent for AVR patients, and 80 per cent (projected) for patients with double valve replacement (85, 96, and 91 per cent for operative survivors, respectively. At this same interval 92 per cent of MVR patients, 99 per cent of AVR patients, and 96 per cent (projected) of patients with double valve replacement were free of thromboembolic episodes. Altogether, 12 of the total 512 valves implanted exhibited some evidence of dysfunction during the entire period of follow-up evaluation, but in only 2 instances (both mitral) was intrinsic pathological involvement of the xenograft tissue documented. Actuarial analysis of xenograft dysfunction at a common interval of 3 years after operation showed 95 per cent of MVR patients, 98 per cent of AVR patients, and 97 per cent (projected) of patients with double valve replacement to be free of this complication. These data support the use of glutaraldehyde-preserved porcine xenografts as superior bioprostheses that pose a low risk of thromboembolism without anticoagulation. The over-all durability of such valves, within the restriction of a maximum current follow-up interval of 4.4 years, appears comparable to that of currently available mechanical prostheses and justifies continued clinical use.*

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Replacement of heart valves with bioprostheses constructed of "fresh" aortic allografts was initiated at Stanford University Medical Center in 1967 because of dissatisfaction with the thromboembolic complications of mechanical valve substitutes then currently available.[1, 2] Subsequent analysis has documented that such allografts are associated with an acceptably low rate of thromboembolism without long-term anticoagulation,[3] but the stability of allograft tissue for either mitral or aortic valve replacement has not proved satisfactory. Five years postoperatively only 40 per cent of patients undergoing mitral valve replacement (MVR) and 49 per cent of patients undergoing aortic valve replacement (AVR) have failed to exhibit clinical and/or pathological evidence of allograft dysfunction of some degree.[4] In most patients who have required reoperation, leaflet degeneration consistent with nonspecific biodegradation has been noted.[3]

We discontinued use of this type of bioprosthesis in 1971 because of the prohibitively high cumulative rate of allograft dysfunction. During the same year we began to utilize another type of composite tissue prosthesis, the glutaraldehyde-preserved porcine aortic valve xenograft.* Experience with this valve substitute had shown that short-term clinical results and valvular

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Volume 73
Number 1
January, 1977



hydraulic function were satisfactory.[5] During the ensuing 4 years porcine xenografts were employed for replacement of mitral and/or aortic valves in 461 patients at this center. The purpose of the present report is to summarize the late postoperative results and to focus particularly upon long-term durability of this bioprosthesis.

## Patients and methods

All patients undergoing MVR and/or AVR with porcine xenografts through July, 1975, are included. For purposes of analysis they are grouped according to the valve(s) replaced, regardless of concomitant operative procedures. Patients undergoing double valve replacement with mixed prostheses (e.g., MVR with a xenograft and AVR with a Starr-Edwards prosthesis*) were excluded from consideration. Selected clinical characteristics of the 243 patients with MVR, 167 patients with AVR, and 51 patients with MVR and AVR are summarized in Table I. Combined procedures such as aorta-coronary bypass grafting, replacement or widening of the ascending aorta, or left ventricular resection were performed in 31 per cent of MVR cases, 43 per cent of AVR cases, and 31 per cent of double valve cases (Table II). It should be noted that during the initial portion of this experience xenograft valves were chosen for implantation primarily in patients in whom long-term anticoagulation was contraindicated; subsequently, as experience with this bioprosthesis accumulated, its use was extended to all patients undergoing valve replacement. Thus not all patients in whom valve replacement was performed at this center during the time period covered are incorporated; however, patients who received xenograft valves did not constitute a significantly select group in terms of clinical characteristics, cause of cardiac disease, or hemodynamic state.

**Operative methods and anticoagulation.** Standard techniques for cardiopulmonary bypass, employing a bubble-type oxygenator, were used. All operations were performed through a median sternotomy. Aortic cross-clamp times for MVR, AVR, and double valve replacement averaged 43, 56, and 93 minutes, respectively. During periods of cross-clamping, local myocardial hypothermia was induced by continuous irrigation of the pericardial sac and intermittent lavage of the left ventricle with cold saline at 3 to 4° C.[6] The commonest valve sizes utilized were 31 mm. (outside diameter) for MVR and 25 mm. for AVR.

Oral anticoagulation with warfarin sodium in standard dosages was prescribed early postoperatively for approximately one third of the patients having either MVR or double valve replacement and for those AVR patients in whom concomitant aorta-coronary bypass grafting had been performed. Subsequently, anticoagulation was discontinued 6 to 8 weeks postoperatively, except at the discretion of the referring physician or in cases of intercurrent thromboembolism. On this basis, 34 patients who underwent MVR remain on a regimen of long-term anticoagulation (25 in atrial fibrillation), as well as 7 patients with AVR and 4 patients with double valve replacement.

**Postoperative evaluation.** Current evaluation of all surviving patients was obtained during a 4 month closing interval (July to October, 1975) by examination at this center or by contact with the patient's physician. Functional classification was assigned on the basis of current symptoms according to New York Heart Association criteria (1964).

* Edwards Laboratories, Inc., Santa Ana, Calif.

**Table I.** *Clinical characteristics of patients undergoing xenograft valve replacement*

| | MVR | AVR | MVR +AVR |
|---|---|---|---|
| Number | 243 | 167 | 51 |
| Sex | | | |
| Women | 150 | 42 | 27 |
| Men | 93 | 125 | 24 |
| Age (yr.) | | | |
| Average | 54 | 58 | 56 |
| Range | 7-74 | 15-84 | 20-77 |
| N.Y.H.A. | | | |
| Class I | 3 | 6 | 0 |
| Class II | 31 | 53 | 10 |
| Class III | 159 | 90 | 34 |
| Class IV | 50 | 18 | 7 |

**Table II.** *Combined procedures during xenograft valve replacement*

| Procedure | MVR | AVR | MVR +AVR |
|---|---|---|---|
| Coronary bypass grafting | 38 | 43 | 7 |
| Ascending aortic graft | 0 | 18 | 5 |
| Septal myectomy | 1 | 4 | 0 |
| Aortic valvuloplasty | 10 | 0 | 0 |
| Mitral valvuloplasty | 0 | 3 | 0 |
| Tricuspid valvuloplasty | 18 | 0 | 3 |
| LV resection/plication | 7 | 0 | 0 |
| ASD repair | 10 | 2 | 1 |
| VSD repair | 2 | 1 | 0 |

*Legend:* LV, Left ventricle. ASD, Atrial septal defect. VSD, Ventricular septal defect.

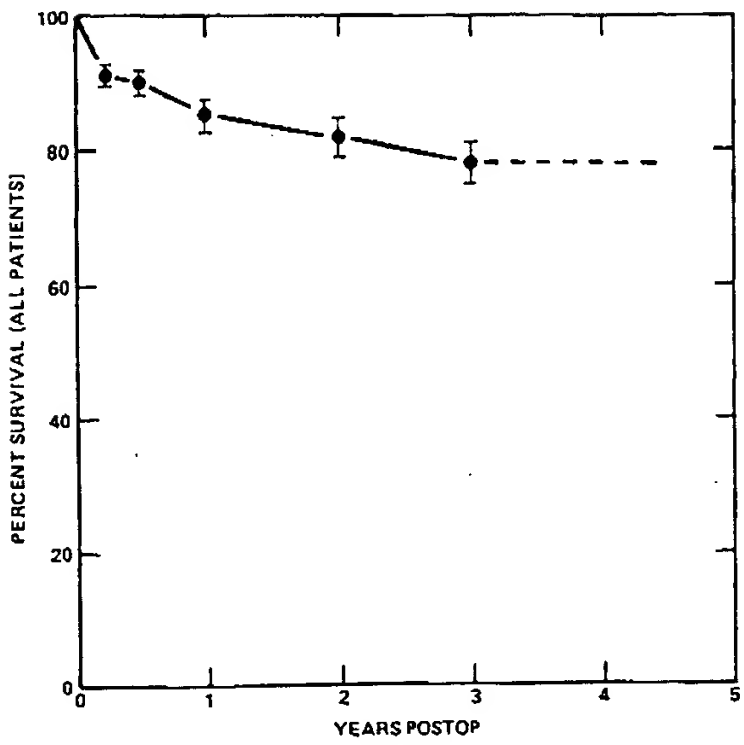


**Fig. 1.** Postoperative survival, calculated by the actuarial method, for all patients undergoing mitral valve replacement with a xenograft. Operative mortality rate is included. The terminal, dashed portion of the survival curve represents an interval during which no event (i.e., death) occurred.

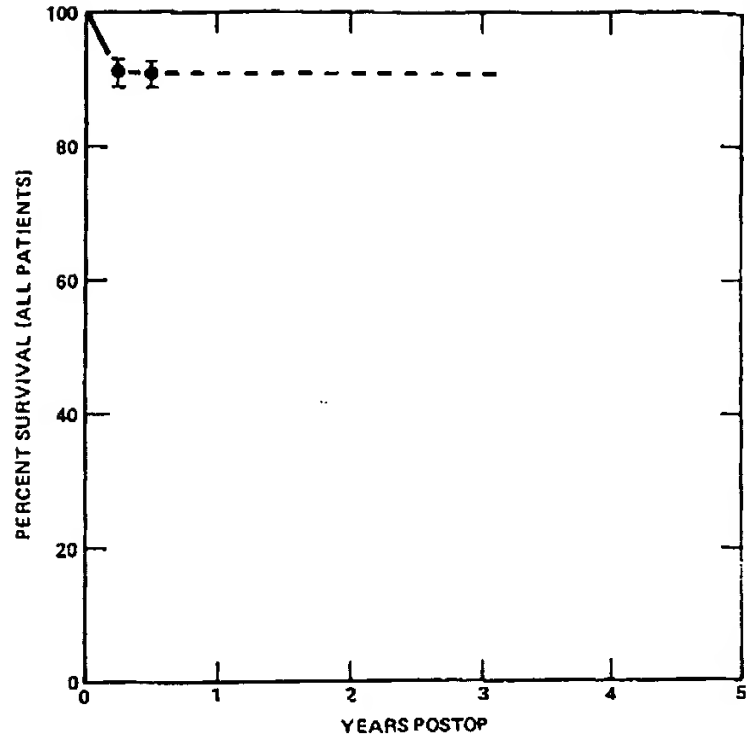


**Fig. 2.** Postoperative survival for all patients undergoing aortic valve replacement with a xenograft. The terminal, dashed portion of the survival curve represents an interval during which no event (i.e., death) occurred.

Xenograft dysfunction was diagnosed on the basis of several criteria: (1) confirmed hydraulic dysfunction sufficient to require reoperation, (2) the postoperative development of a new murmur characteristic of valvular regurgitation (aortic or mitral), unless proved by angiocardiography to represent solely a periprosthetic leak without any central component involving the xenograft leaflets, or (3) endocarditis resulting in death or reoperation. The second criterion is considered necessary inasmuch as a newly developed regurgitant murmur after tissue valve replacement cannot reliably be distinguished clinically as representing perivalvular versus transvalvular insufficiency, in contrast to experience with most mechanical valve substitutes.

Thromboembolic events were categorically diagnosed on the basis of any new neurologic deficit, whether transient or permanent, or other peripheral arterial embolic episode. Only cerebrovascular accidents proved by postmortem examination not to have resulted from emboli were excluded from classification as emboli. Patients were considered at risk for thromboembolism throughout the current period of follow-up or until death or reoperation.

The total duration of follow-up for patients with MVR was 347 patient-years (summation of all individual follow-up intervals, whether to death or current evaluation); the average follow-up interval for surviving patients was 1.6 years (range 1 month to 4.4 years). For patients with AVR the total follow-up analysis included 148 patient-years (average for surviving patients 1.0 year, range 1 month to 4.0 years), and for patients with MVR and AVR the total duration of follow-up was 37 patient-years (average follow-up for surviving patients 0.9 year, range 1 month to 2.4 years).

The total duration of follow-up in patient-years for each of the three groups was used as a denominator for calculation of *linearized* rates of specific events such as thromboembolism or xenograft dysfunction. The total number of such events was divided by the total number of patient-years of follow-up and multiplied by 100 to express average risk as per cent per patient-year. Alternately, this calculation may be expressed as number of events per 100 patient-years. Although this method of risk calculation provides a realistic approximation of statistical probability for some events, it may overestimate individual risk for some complications, such as thromboembolism, either because of multiple events sustained by a single patient or because of "clustering" during the early postoperative period in a patient group with limited duration of follow-up. Rates of complications and survival, therefore, were also calculated by the actuarial method that provides an estimate of the per cent of the total patient group free of or affected by an event over time. This implies withdrawal of a pa-

Volume 73
Number 1
January, 1977



tient from further consideration after an initial event such as thromboembolism (or after the available follow-up interval). Actuarial curves were also used to assess the impact of a single variable after a group of patients under consideration had been dichotomized on the basis of this variable. A two-tailed significance level was assigned to the p values thus obtained.

## Results

**Operative mortality rates.** Over-all operative mortality rates (before hospital discharge) for MVR, AVR, and double valve groups were 7.8, 6.0, and 11.8 per cent, respectively. No death was attributable to xenograft dysfunction. Approximately 60 per cent of all early postoperative deaths were due to myocardial failure (two secondary to myocardial infarction) and the remainder to diverse causes such as sepsis, renal failure, cerebrovascular accidents, or complications categorized as technical (ruptured hematoma of the posterior atrioventricular groove after MVR in 2 patients and delayed rupture of an aortic pericardial gusset in one patient).

**Late postoperative survival.** Long-term postoperative survival rates for the MVR and the AVR groups are illustrated by the actuarial curves shown in Figs. 1 and 2. In the former group the survival rate for all patients 4.4 years postoperatively (maximum follow-up interval) was 78 (±3.8) per cent; for operative survivors, it was 85 (±3.8) per cent. Seventeen of the 21 late deaths (81 per cent) were cardiac in origin. Most of these were due to congestive heart failure or myocardial infarction; three were sudden and unexplained. No patient died of diagnosed arterial embolization, but one patient who was receiving oral anticoagulation died of intracerebral hemorrhage. One late death was indirectly related to xenograft failure (to be discussed); in this instance, reoperation for a periprosthetic leak that had developed after previous reoperation for idiopathic perforation of a xenograft leaflet was unsuccessful. The remaining late deaths were due to causes unrelated to cardiac or xenograft function.

MVR patients with associated coronary artery disease accounted for a substantial portion of both early and late deaths. For example, in 54 patients the diagnosis of coronary artery disease was established by coronary angiography, electrocardiographic evidence of previous myocardial infarction, or postmortem examination within one year after operation. Operative mortality rate in this total group was 18.5 per cent, and the survival rate at 2 years was only 56 (±7.8) per cent. Patients undergoing simultaneous coronary artery bypass grafting for angiographically defined coronary artery lesions fared similarly. In contrast, patients with mitral valve dysfunction not associated with overt coronary artery disease (189 patients) sustained an operative mortality rate of 4.2 per cent and exhibited a 3 year survival rate of 87 (±3.0) per cent (91 ± 3.3 per cent for operative survivors). These differences in survival between patients with and without associated coronary artery disease were highly significant ($p < 0.001$).

Late postoperative survival after xenograft AVR, illustrated in Fig. 2, was 90.6 (±2.3) per cent at 3 years. Postoperative follow-up in this group extends to a maximum of 4 years, but one late death at 3.2 years is not graphed because of an excessive standard error (±32 per cent) that precludes meaningful analysis at this interval. Discharged patients exhibited a survival rate of 96 (±1.6) per cent at 3 years. Three of the six late postoperative deaths were due to congestive heart failure and one to myocardial infarction; one was caused by endocarditis (to be discussed) and one by pulmonary embolism. Despite the small number of late deaths after AVR available for analysis, correlation of over-all survival with specific etiologic diagnoses revealed that the survival rate for patients with pure aortic regurgitation secondary to degenerative connective tissue disorders or obscure causes (e.g., mucinous degeneration with valvular prolapse) (18 cases) was significantly inferior to that of the remaining patients.

Comparison of patients with coronary artery disease documented by angiography preoperatively (47 cases) with those in whom significant coronary narrowing was proved absent by arteriography (61 cases) showed a higher operative mortality rate for the former group (8.5 versus 3.3 per cent) but equal numbers of late deaths (two in each group); because of the small total number of lethal events, however, the difference in over-all survival was not statistically significant. Nevertheless, the survival rate of patients with angiographically demonstrated coronary artery disease who underwent coronary bypass grafting simultaneously with AVR (37 cases) was slightly superior to that of patients in whom coronary bypass grafting was omitted (10 cases) ($p' = 0.07$, two-tailed analysis).

Three late postoperative deaths have occurred after double valve replacement. One was due to congestive heart failure and the other two to noncardiac causes. Maximum follow-up in this group is limited to 2.4 years. The survival rate at 2 years for all patients was 80 (±6.3) per cent and that for discharged patients was 91 (±5.3) per cent. Patients with associated coronary artery disease had over-all inferior survival rates, but too few patients underwent simultaneous coronary

**Table III.** *Causes of diagnosed xenograft dysfunction*

| *Cause* | *MVR (n =243)* | *AVR (n =167)* | *MVR +AVR (n =51)* |
|---|---|---|---|
| Insufficiency murmur | | | |
| Early | 4 | 1 | 1 |
| Late | 1 | 0 | 0 |
| Endocarditis | 1 | 1 | 0 |
| Valve stenosis | 0 | 1 | 0 |
| Primary tissue involvement | 2 | 0 | 0 |
| Totals | 8 | 3 | 1 |

bypass grafting to assess the influence of this combined procedure.

**Xenograft dysfunction.** A diagnosis of xenograft dysfunction was established for 12 of the total 512 valves implanted in the 461 patients, eight in the MVR group, 3 in the AVR group, and one (aortic) in a patient who had undergone double valve replacement. As summarized in Table III, the basis for diagnosis of dysfunction was a murmur of valvular regurgitation in 8 of these instances; in 6 this was detected within the first 3 postoperative months. In the remaining 2 patients murmurs were first noted more than one year after MVR: One of these patients remains asymptomatic and the cause of valvular regurgitation has not been determined. The other patient required subsequent reoperation, and a 5 mm. perforation near the leading edge of one of the xenograft leaflets was found; no cause for this defect could be defined by microscopic examination. The other leaflets of this xenograft valve had no abnormalities.

In 2 patients, documented xenograft stenosis sufficiently severe to require reoperation constituted the basis for diagnosis of valvular dysfunction. One valve, a 23 mm. (outside diameter) aortic prosthesis, showed no intrinsic abnormality. In the other case, however, tissue overgrowth of the atrial aspect of a 27 mm. mitral substitute had rendered the xenograft leaflets immobile.

The remaining 2 instances of xenograft dysfunction were due to endocarditis (one mitral, one aortic). Both patients underwent reoperation that was unsuccessful.

Thus two of the total 12 valves categorized as dysfunctional in this anaylsis have been documented as presenting features of primary involvement of xenograft tissue (both in the mitral location). In the remainder, the cause of dysfunction remains unknown or was secondary to infective lesions. A total of five reoperations were performed because of intrinsic or infective valve dysfunction.

Actuarial analysis of these data indicates that up through the maximum periods of follow-up 95 per cent of patients with MVR, 98 per cent of patients with AVR, and 97 per cent of patients with double valve replacement are free of evidence of xenograft dysfunction. Linearized rates of valve dysfunction for these three groups are 2.3, 2.0, and 2.7 per cent per patient-year, respectively.

**Thromboembolism.** Seventeen patients undergoing MVR sustained a total of 18 postoperative cerebral or peripheral vascular events categorized as emboli—a linearized thromboembolism rate of 5.2 per cent per patient-year. Actuarial analysis indicates 92 (±2.0) per cent of patients to be free of emboli throughout the period of follow-up (4.4 years). There was no significant difference in the time-related incidence of thromboembolism in patients dichotomized on the basis of preoperative or current cardiac rhythm (i.e., sinus rhythm versus atrial fibrillation). Similarly, long-term anticoagulation conferred no detectable protection against emboli. It is noteworthy, however, that 76 per cent of all diagnosed emboli occurred within 2 months of operation.

In the AVR group three embolic episodes were diagnosed (3 patients), two within the first postoperative month. The linearized rate of thromboembolism is thus 2.0 per cent per patient-year, and 99 (±0.8) per cent of patients are predicted to be free of emboli throughout 3 years of follow-up. The third neurologic event occurred subsequent to 3 years postoperatively and is not incorporated into this actuarial calculation because of a standard error of ±27 per cent associated with the cohort at this interval.

Two embolic episodes were diagnosed in 2 patients undergoing double valve replacement, both within the first postoperative month. These two events yield a linearized thromboembolism rate of 5.4 per cent per patient-year and an actuarially predicted embolus-free proportion of 96 (±2.9) per cent throughout the period of follow-up.

**Current status.** Currently, 91, 96, and 93 per cent of patients discharged after MVR, AVR, and double valve replacement, respectively, are living. The majority enjoy greatly improved physical capacity as compared to preoperative disability. Sixty-four per cent of MVR patients, 67 per cent of AVR patients, and 57 per cent of MVR plus AVR patients are categorized in Functional Class I. Nearly all of the remaining patients are in Functional Class II; only 10 of the total 396

Volume 73
Number 1
January, 1977



surviving patients retain Functional Class III or IV disability.

## Discussion

Various types of tissue for fabrication of heart valve substitutes have been evaluated by many investigators over the past several years.[7–13] A primary stimulus for the development and testing of bioprostheses has been dissatisfaction with the combined risks of thromboembolism and oral anticoagulation that are associated with mechanical prostheses. For example, analysis of our institutional experience has shown the linearized thromboembolism rate for mitral valve replacement with the Starr-Edwards Model 6120 valve (515 patients) to be 10.9 per cent per patient-year and for aortic valve replacement with Models 1200 or 1260 (639 patients) to be 6.0 per cent per patient-year (there was no significant difference in this regard between the latter two aortic prostheses). Furthermore, in the same patient groups the linearized morbidity rate for long-term anticoagulation (hemorrhagic episode) was 5.5 per cent per patient-year after mitral valve replacement and 5.1 per cent per patient-year after aortic valve replacement; linearized mortality rates associated with oral anticoagulation were 0.9 per cent per patient-year and 0.7 per cent per patient-year, respectively. It is recognized that morbidity and mortality rates reflecting the risk of long-term anticoagulation are influenced importantly by the quality of anticoagulation control, but information regarding such is not available for our experience. It is furthermore recognized that subsequent design changes in mechanical prostheses have resulted in thromboembolism rates that are generally reported to be significantly reduced, as compared to the valve models just described.[14–16] A continuing need for indefinite oral anticoagulation, however, has been maintained.

To varying degrees the objective of achieving decreased thrombogenicity with valves of biological origin has been realized. An important disadvantage of many such bioprostheses, however, exposed only after long-term observation and evaluation, has been limited durability. Valves fabricated of undifferentiated autologous or homologous tissue such as untreated pericardium[17] or fascia lata[12] have exhibited prohibitively high failure rates. Allograft aortic valves processed by chemical sterilization or preservation techniques that cause total loss of cell viability have shown high rates of pathological deterioration over the first few years after implantation.[18–20] The use of "fresh" allograft aortic valves at this institution was predicated in part upon the theoretical expectation that viability of cells in the allograft leaflets would contribute to maintenance of structural valve integrity. As noted previously, however, the late postoperative results of this experience have failed to substantiate stability of this type of bioprosthesis: 5 years after implantation 60 per cent of patients undergoing MVR and 51 per cent of patients undergoing AVR have exhibited some evidence of allograft dysfunction.[4] Because of these prohibitively high dysfunction rates, use of aortic valve allografts was discontinued.

An alternate approach to achieving long-term biostability of tissue valves has consisted of preoperative treatment with aldehyde agents to cross-link collagen molecules and thus render the major tissue components biostable. Evaluation of xenograft aortic valves treated by formaldehyde solutions, however, has shown that leaflet tissue preserved by this technique is biodegradable and thus the rates of valve failure are high.[21]

More encouraging clinical results have been obtained with glutaraldehyde preservation of porcine xenograft valves.[5, 22, 23] The intermolecular bonds generated by this agent appear to be biostable, and longterm flexibility of the leaflets is maintained. An additional theorectical advantage of the glutaraldehyde-preserved porcine xenograft utilized in our experience is flexibility of the tricornute stent, a feature that has been demonstrated in vitro to reduce maximum tissue stress at the leading edges of the valve cusps under loading conditions[5]: Whether this potentially advantageous property of the valve in fact contributes to functional valve longevity is presently unknown.

Our[22] early experience with this type of bioprosthesis for MVR produced satisfactory clinical results in terms of restoration of physical capacity and an acceptably low rate of thromboembolism without long-term anticoagulation. The present analysis of the results of both MVR and AVR, incorporating longer periods of postoperative evaluation extending to nearly 4.5 years after mitral valve substitution, confirms these earlier observations. Throughout the intervals of follow-up available, 92 per cent of MVR patients, 99 per cent of AVR patients, and 96 per cent of patients undergoing double valve replacement remain free of thromboembolic episodes. Only a small minority of patients have been maintained on a regimen of oral anticoagulation for individual reasons at the discretion of the patient's private physician. In the case of MVR, calculation of the time-related incidence of thromboembolism slightly exceeds that reported by Bonchek and Starr[14] for

cloth-covered mechanical prostheses (1.9 per cent per patient-year, 97 per cent embolism-free rate at 5 years); indefinite oral anticoagulation, however, is required for the latter valves, and the attendant risks must be taken into consideration in over-all comparative analysis.

The low over-all incidence of xenograft valve dysfunction revealed by this evaluation of 512 valves in 461 patients justifies, we believe, characterization of this bioprosthesis as superior to biological valve substitutes previously used widely. Altogether, 12 instances of xenograft dysfunction were diagnosed, including insufficiency murmurs of unknown cause, endocarditis resulting in death or reoperation, intrinsic valve stenosis requiring reoperation, and tissue deterioration. In only 2 instances, however, was intrinsic degeneration of xenograft leaflets documented. In one case this resulted from tissue overgrowth of the inlet orifice of a mitral substitute, and in the other it consisted of idiopathic perforation of a single xenograft leaflet.

Indeed, the stability of xenograft structure and function would appear comparable to that of currently available mechanical prostheses, if all causes of valvular instability are taken into account. Maximum follow-up evaluation of xenograft valves now extends to nearly 5 years, and, within the constraints of this interval, such valves appear sufficiently biostable to warrant their continued clinical use.

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Volume 73
Number 1
January, 1977



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## Discussion

### DR. ALBERT STARR

*Portland, Ore.*

Dr. Stinson was kind enough to write to me before this meeting so that I could have some slides prepared which would be similar, to some extent, in comparing the results with the heterograft prosthesis to results with the non–cloth-covered and the cloth-covered ball valves.

The figures that I have taken for the xenograft were from the abstract and are not exactly the same as those presented in the paper, but, they are quite close. For mitral valve replacement with the xenograft, the 3 year actuarial survival rate was 85 per cent; in our own series, this rate was 88 per cent for the non–cloth-covered valve and 86 per cent for the cloth-covered valve. At 6 years, there is no information concerning the xenograft; the 6 year survival with the non–cloth-covered ball-valve prosthesis was 78 per cent, and with the cloth-covered valve, 72 per cent. At 10 years, we have information only for the non–cloth-covered valve, the actuarial survival rate being 61 per cent.

If one considers the thromboembolism rate, the xenograft actually provides no significant improvement over the non–cloth-covered valve, and it is not quite as athrombogenic as the cloth-covered valve for mitral valve replacement in anticoagulated patients. The incidence of thromboembolism with the xenograft was approximately 5 per cent per year, versus 6 per cent per year for the non–cloth-covered valve in the mitral position and 3 per cent per year for the cloth-covered valve in the mitral position. Again, I stress that patients with these ball valves have had long-term anticoagulant treatment.

For aortic valve replacement, the embolic rate is 2 per cent per year for the xenograft and 1.6 per cent per year for cloth-covered aortic valve with anticoagulants. There is no statistically significant difference.

Admittedly, this comparison will not be completely fair, since the follow-up is so short for the xenograft. There may be clustering of early embolic complications in the first year of follow-up, which would tend to skew the information against the xenograft prosthesis.

With the track valves, however, the longest and mean follow-up is the same as with the xenograft, and this series is more comparable. We can see no significant difference in the results in terms of survival and embolic incidence at the end of 3 years with anticoagulant therapy. For example, the 3 year survival rate with the mitral xenograft is 85 per cent and with the track valve in the mitral position, 89 per cent. Ninety-two per cent of patients with the mitral xenograft were free of embolism, versus 89 per cent of those with the mitral track valve (figures determined actuarially).

With regard to survival, following aortic valve replacement, the results are the same: 96 per cent versus 97 per cent. The embolism-free rate at 3 years was 99 per cent versus 97 per cent.

On the basis of this information, we continue to use both the cloth-covered valve in the track configuration and the non–cloth-covered valve. We use the heterograft prosthesis in cases in which anticoagulation is not possible. We all will surely follow, with great interest, the longer term results in Dr. Stinson's series of patients to see what the truly late complication rate will be.

### DR. ALAIN CARPENTIER

*Paris, France*

I would like to congratulate Dr. Stinson on his excellent and well documented presentation. Dr. Shumway's group at Stanford has been pioneering in tissue valves for a long time, and any statement from this group deserves particular attention.

I shall limit my comments to the major problem raised by the xenograft valve, which is its durability. Since we introduced the use of glutaraldehyde for xenograft preservation in 1968, we now have an 8 year follow-up. In a first period of clinical investigation (1968 to 1970), we used two methods of glutaraldehyde preservation: buffered glutaraldehyde 1 per cent in the first 11 patients and glutaraldehyde 0.6 per cent plus sodium metaperiodate in a following series of 91 patients. Because the incidence of valve failure was higher in the metaperiodate glutaraldehyde group, we subsequently used buffered glutaraldehyde without metaperiodate. Valve failures in both groups were of three types: infection, calcification, and tissue perforation or tears. The third complication was the result of either localized inflammatory reaction or fatigue lesions.

[Slide] This slide shows an example of calcification one year after implantation in the pulmonary position.

[Slide] This slide shows a valve perforation due to fatigue lesions at 6 years.

It should be emphasized that most of these failures occurred in valves prepared in our research laboratory before the valve became commercially available. The incidence of valve failure has been reduced in the past 4 years by using valves prepared on a large scale by better manufacturing facilities with superior quality control.

The main concern remains the tissue fatigue lesions. On a specimen removed at 6 years, despite excellent hemodynamic function, we found areas of collagen degeneration and elastic fiber fragmentation. The tissue was slightly thinner than before implantation. On the other hand, the cusp surface was covered by host fibrous tissue. This process of encapsulation is different from that of host cell ingrowth seen in valvular grafts, since there is no cell penetration into the tissue. This process could be of critical importance for the long-term durability of these bioprostheses as the valve structure is itself reinforced.

Thus at least four factors may play a role in the long-term durability of a bioprosthesis: tissue preservation, use of a flexible stent, valve selection and mounting, and the process

of encapsulation. We can influence these factors, and we are continuously at work to do so. However, we must remember that, owing to the nature of the bioprosthesis itself, progressive fatigue alteration of the valve tissue is inevitable.

The currently available models should last 10 years, with a 15 to 20 per cent incidence of valve failure. This represents a significant advance as compared to the 50 per cent failure rate at 2 years with our first method of preservation with a mercurial salt (1965 to 1966) and the 50 per cent failure rate at 4.5 years with the formalin-treated grafts (1966 to 1968). As confirmed by Dr. Stinson, valvular xenografts are now competitive with mechanical valves. My present feeling is that xenograft valves complement mechanical valves, giving the patient and his physician the choice between an improved quality of life with a bioprosthesis versus a lower risk of reoperation with a mechanical valve.

## DR. VINCENT GALLUCCI

*Padua, Italy*

At the University of Padua Medical School, 550 patients have had one or more cardiac valves replaced with the Hancock xenograft by one of two surgical groups. This xenograft was placed in the mitral or aortic position, or both, in some patients and in the tricuspid position in others. I want to give a brief report on our results up to December, 1975, as they are a bit different from those described by Dr. Carpentier with regard to durability.

We started about 7 years ago with the first small series of formalin-treated xenografts. The glutaraldehyde-preserved valve, which has remained unchanged since November, 1969, has been used for the past 6 years.

In March, 1969, we began implanting 40 formalin-fixed porcine bioprostheses, most of them in the mitral position. The great majority of these failed and had to be replaced, although 5 of them are still functioning well, which is difficult to understand indeed. It is common knowledge that formalin-fixed tissue valves gave very unsatisfactory results universally.

Our two surgical groups implanted glutaraldehyde-processed Hancock xenografts in 441 patients up to the end of 1975.

[Slide] This slide shows an actuarial survival curve for mitral valve replacement, which also comprises the hospital mortality rate. It seems to me very significant that, from the first year on, the curve is on a straight horizontal line for the next 5½ years.

[Slide] This table summarizes the late complications. In 387 patients there were 6 episodes of thromboembolism, 2 cases of prosthesis failure, 3 of endocarditis, and one case of mitral valve restenosis.

[Slide] Here is a functional classification of the long-term survivors with Hancock xenografts in the mitral position. Their present status seems to be quite good, as confirmed by several hemodynamic controls. Most of the patients changed from N.Y.H.A. Classes III and IV to Classes I and II.

In conclusion, 2 years ago, in discussing Dr. Carpentier's paper at the Association meeting, I stated that our policy was to use xenograft valves in selected cases only, mainly in young women still willing to bear children or in those patients not suitable for anticoagulant therapy.

At the present time, because of our long-term clinical results and of the extremely low incidence of thromboembolism and valve dysfunction, I believe it justified to say that the glutaraldehyde-preserved Hancock xenograft has passed the test of time. We recommend it for just about any patient.

## DR. SIDNEY LEVITSKY

*Chicago, Ill.*

I rise to report a complication of the Hancock porcine prosthesis that may be a forerunner of future problems. A porcine valve was removed from a 9-year-old child at the University of Illinois Medical Center. The patient had had mitral valve replacement in March, 1973, for severe mitral insufficiency after having undergone a mitral valvuloplasty 9 months previously by another surgical group. The child was recatheterized on a routine basis 12 months following valve replacement with the porcine prosthesis. All resting pressures were normal, and angiography revealed a normal, functioning valve.

Seventeen months postoperatively, the patient became symptomatic. A second postoperative catheterization at 19 months revealed severe mitral stenosis and prompted reoperation.

Examination of the valve that was removed revealed that all the cusps were stiff; there were areas of calcification on both the atrial and ventricular surfaces. Histologic sections were reviewed by Dr. William Roberts at the National Institutes of Health and by Dr. Maurice Lev of Chicago, who agreed that there was evidence of a wearing or aging phenomena involving the valve and contributing to its degeneration. There was no evidence of rejection or endocarditis.

At the present time, we do not understand (and perhaps Dr. Stinson can answer this for us) why this patient's valve underwent accelerated degeneration when none of our other patients over the past 4 years have exhibited this phenomenon. Moreover, I am concerned that as we accumulate more lengthy experience, this problem may be the rule rather than the exception.

Nevertheless, with the present state of the art of prosthetic valve production, the Hancock porcine valve prosthesis currently remains our first-line valve.

## DR. STINSON *(Closing)*

I appreciate very much Dr. Starr's clear and detailed comments. Inter-institutional comparisons are dangerous, at best, but nevertheless must be based upon at least similar statistical methods of calculation. He has described basically no significant differences within the constraints of the follow-up intervals available for survival and for the risk of thromboembolism, the exception being that the mechanical prosthesis does require long-term anticoagulation. This factor must be taken into account in any long-term comparative analysis of clinical utility.

For instance, in our evaluation of the long-term results with

Volume 73
Number 1
January, 1977



about 1,200 patients, contributing nearly 5,000 patient-years for analysis, who had undergone replacement with the Starr-Edwards Model 6120, 1200, or 1260 valves, I analyzed morbidity and mortality rates for anticoagulation. The average morbidity rate was 5.3 per cent per patient-year; that is, for an hemorrhagic episode. The mortality rate averaged 0.8 per cent per patient-year. This consideration must be taken into account in comparative analysis.

Dr. Starr was also correct in emphasizing that the clustering of events during an early portion of the follow-up interval is a restrictive factor in utilizing linearized rates of thromboembolism, or any other such event, in patients with a limited duration of follow-up.

Dr. Carpentier is recognized to be a pioneer in this field. It was quite appropriate for him to emphasize differences in the types of chemical treatment of tissue for long-term implantation. Biochemically, there is a gross difference between a process that incorporates a previous oxidation step with sodium metaperiodate and one which does not, before treatment with glutaraldehyde. His word of caution regarding the long-term outlook is appropriate. I cannot respond to his comments on the basis of our data, inasmuch as two valve failures in a total experience of 532 patient-years simply do not provide a meaningful data base for long-term extrapolation. .

Dr. Levitsky, in contrast to your conclusion, I would submit that your case represents the concept that the exception does indeed prove the rule.

# The Angell-Shiley Porcine Xenograft

William W. Angell, M.D., Judith D. Angell, B.S., Alex Sywak, M.D.

ABSTRACT **A 4-year clinical experience with fresh allograft tissue valves prompted a trial of 0.5% buffered glutaraldehyde as a valve fixative and sterilant. Tanned allograft and porcine xenograft valves were inserted into experimental animals, and, beginning in 1970, similar valves were implanted in a series of patients now totaling 312. The clinical results are excellent. The 5-year valve-related mortality is 6% for patients who had mitral valve replacement and 16% for those with aortic valve replacement. To date, the incidence of thromboembolism is 1.3% per patient-year, and valve-related morbidity and mortality for the combined groups is 27.4%.**

**Valve stent design has evolved from symmetrically configured metal to anatomically molded plastic. The maintenance of natural valve configuration has optimized leaflet coaptation and support, decreased tissue stress, and eliminated valve-stent dehiscence and tissue rupture seen in valves deformed to fit symmetrical stents. Stent design, controlled glutaraldehyde solutions, and fixation techniques have improved leaflet flexibility and reduced valve orifice to annulus diameter ratios, thus producing transvalvular gradients comparable to both mechanical and modified orifice tissue valves. To date, tissue failure, observed in only 1.0% (3 of 287) of patients, is the result of calcification (2 patients) and cusp rupture due to incomplete fixation (1 patient).**

Interest in the development of a xenograft prosthesis was stimulated by the logistical problems of availability, guaranteed sterility, and limited storage which plagued an otherwise favorable 3.5-year clinical experience with the fresh allograft [4]. Because of the complete failure of 33 formalin-treated porcine valves [8], the use of glutaraldehyde as a fixative and sterilant was approached cautiously. Animal trials were followed by a small clinical series of 6 patients in 1970 [1]. Careful follow-up of these patients over a 2½-year interval revealed no evidence of valve failure or complications [5]. The decision was made to transfer the knowledge and experience gained from the use of both the autograft and the formalin xenograft to the fabrication of a glutaraldehyde tanned porcine valve graft. Since then, the association with Shiley Laboratories has added a further dimension of expertise to the development of this tissue valve prosthesis. This report involves 331 glutaraldehyde-treated xenografts implanted in 312 patients between June, 1970, and October, 1978. The series is analyzed with regard to overall patient morbidity and mortality, including the incidence of tissue failure, thromboembolism, bacterial endocarditis, anticoagulant-related hemorrhage, and hemodynamic performance. The results of this analysis support the impression that the glutaraldehyde-treated xenograft provides extended durability with a low incidence of valve-related complications.

## Material and Methods

Between June, 1970, and October, 1978, 312 patients underwent aortic (AVR), mitral (MVR), or multiple valve replacement utilizing 331 glutaraldehyde-treated porcine xenografts (Table 1). There were 227 men and 85 women with an average age of 55 years (range, 21 to 83 years old). Twenty-nine percent of the patients were 65 years old or older. As shown in Table 2, 72% of the patients had valve replacement as an isolated procedure, while the remaining 28% (86) underwent valve replacement in combination with a second or third surgical procedure, usually coronary artery bypass grafting. Fifty-six patients (18%) underwent valve replacement with a porcine xenograft because of fail-

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Supported in part by Grant No. 75–944 from the American Heart Association and by Research Funds from the Veterans Administration Medical Center, Palo Alto, CA.

Presented at the Twenty-fifth Annual Meeting of the Southern Thoracic Surgical Association, Nov 2–4, 1978, Marco Island, FL.

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*Table 1. Patient Information*

| Factor | AVR | MVR | Mult VR |
|---|---|---|---|
| No. of patients | 176 | 94 | 42 |
| Men | 150 | 54 | 23 |
| Women | 26 | 40 | 19 |
| Average age (yr) | 53 | 56 | 55 |
| Range (yr) | 21–83 | 21–79 | 29–75 |
| 65 years or over | 32% | 23% | 26% |
| Follow-up | | | |
| Duration (patient-years) | 417 | 221 | 69 |
| Average (yr)[a] | 2.5 | 2.6 | 2.1 |
| Maximum (yr) | 8.1 | 8.3 | 5.3 |
| Range (yr) | 0.1–8.1 | 0.1–8.3 | 0.2–5.3 |
| Lost | 1.7% | 0 | 0 |
| Current survivors | 147 | 67 | 23 |

[a]Patients surviving operation.
AVR = aortic valve replacement; MVR = mitral valve replacement; Mult VR = multiple valve replacement.

*Table 2. Patient Groups*

| Procedure | AVR | MVR | Mult VR |
|---|---|---|---|
| Total patients | 176 | 94 | 42 |
| Isolated procedure | 121 | 68 | 37 |
| Combined procedure | 55 (31%) | 26 (28%) | 5 (12%) |
| CABG × 1 | 24 | 12 | 0 |
| CABG × 2 | 19 | 11 | 3 |
| CABG × 3 | 3 | 3 | 0 |
| Pacemaker | 7 | 0 | 2 |
| VSD closure | 2 | 0 | 0 |
| Previous valve operation | 24 (14%) | 18 (19%) | 14 (33%) |

AVR = aortic valve replacement; MVR = mitral valve replacement; Mult VR = multiple valve replacement; CABG = coronary artery bypass graft; VSD = ventricular septal defect.

ure of a previous graft. Only 37% of patients were considered ideal surgical candidates: they were less than 65 years old, had a cardiothoracic ratio of less than 0.55, and were undergoing a first procedure for isolated valve replacement.

All patients seen with operable valvular heart disease were considered candidates for valve replacement with a porcine xenograft. There was no patient selection by age or by New York Heart Association Functional Classification. The patients in this series are, for the most part, consecutive, and tissue valves were used exclusively unless there was a patient or physician preference. All patients receiving xenografts are included in this analysis with the exception of 4 patients (3 undergoing MVR and 1, AVR) who were operated on for primary coronary artery disease and in whom a valve was inserted only as a final attempt to wean the patient from cardiopulmonary bypass. All 4 patients died in the operating room.

### *Operative Technique*

Both AVR and MVR are performed through a median sternotomy. Standard cardiopulmonary bypass with caval and ascending aortic root cannulation was used in all patients but those undergoing single aortic valve replacement; in those patients, a single right atrial cannula was used. Myocardial protection during aortic cross-clamping has evolved from surface cooling or coronary perfusion to the present use of cardioplegic solutions combined with surface and intracavitary cooling. Suturing methods have remained unchanged for MVR. Interrupted mattress Teflon-coated Dacron sutures from the ventricle to the atrium are used to secure the valve in place. Teflon bolsters are used in areas with minimal annular fibrous tissue. The valve is positioned as high in the left atrium as possible, and it is rotated to ensure placement of the widest valve sinus in the area of the left ventricular outflow tract. The technique for AVR has changed from intraannular positioning of the valve with interrupted mattress sutures from the aorta to the ventricle to supraannular positioning with sutures from the ventricle to the aorta [1]. Also, the valve is rotated so as to align the commissural strut between the right and left coronary sinuses of the valve midway between the right and left coronary ostia of the patient. This places the muscular right coronary cusp of the valve against the anterolateral muscular ridge of the left ventricle and positions the larger, less obstructive left valve cusp and the noncoronary valve cusp in the widest portion of the left ventricular outflow tract. In some instances, patch widening of the aortic root is used, but rarely is the annulus split [24]. These alterations in AVR

technique are thought to allow the use of larger valves and to reduce transvalvular gradients.

*Anticoagulation*

Postoperatively, most patients are treated with heparin, 50 to 75 U per kilogram of body weight administered twice a day beginning approximately eighteen hours after operation, and with warfarin until the prothrombin time reaches 20 to 25% (2 to 2.5 times normal). Then, heparin is discontinued, and the prothrombin time is maintained at this level with warfarin for 8 to 16 weeks, after which it is gradually withdrawn by tapering doses over an 8-week period. Patients in atrial fibrillation with a history of thromboembolism and patients who, in addition, were found to have left atrial clot, intimal disruption, or a "giant" left atrium at the time of operation were recommended for long-term warfarin therapy. Patients with a history of hemorrhage on a regimen of anticoagulants or in whom it was difficult to control the prothrombin time were placed on abbreviated or modified regimens of anticoagulation.

*Analytical Methods*

Follow-up was conducted over a 2-month interval by contacting the patient or the referring physician or both parties. Patients were asked to complete a standard questionnaire specific for the type of valve replacement and designed to determine the presence of a valve-related complication or the risks of such a complication. When a complication was suspected or known to have occurred, the referring physician was contacted for verification and clarification or the patient was seen in this office.

Table 1 summarizes the follow-up information for each valve replacement group. The total duration of follow-up for all patient groups is 707 patient-years, with an average of 2.5 years and a range of 0.1 to 8.3 years. Of the 287 patients who survived operation, 3 patients (1%) who underwent AVR have been lost to follow-up.

Designation of patient morbidity and mortality as operative, valve-related, or non-valve-related was based on the following:

1. All deaths within thirty days of operation or related to events within the first thirty days were designated as operative deaths. All patients who died of valve-related causes within thirty days of operation are included in the valve-related morbidity and mortality curves.
2. All sudden, unexplained deaths are designated as valve-related.
3. All cerebral vascular accidents and transient ischemic attacks are designated as valve-related.
4. All bacterial endocarditis was designated as valve-related, even in patients with multiple-valve replacement in whom a mechanical prosthesis or allograft valve was also present.
5. All hemorrhages occurring when the patient was on a regimen of anticoagulants for the valve were designated as valve-related.
6. All reoperations were designated as valve-related.
7. Hemodynamically significant regurgitation or stenosis as determined by clinical and catheterization methods was designated as valve-related.
8. Questionable or hemodynamically insignificant murmurs were not considered valve-related complications.
9. Hepatitis was not included as a valve-related complication.

Patient morbidity and mortality, overall and valve-related, are expressed both by standard actuarial survival curves and by linearized occurrence rates (Table 3) [6, 11, 16].

*Patient Survival*

The hospital mortality for xenograft AVR is 4%. Overall late mortality expressed as a linearized rate is 6.5% per patient-year (see Table 3). The actuarial overall survival curve* for AVR shows 68% of patients alive at 8 years (Fig 1). When deaths from non-valve-related causes are excluded, the linearized risk of late valve-related mortality is 3.4% per patient-year, and the ac-

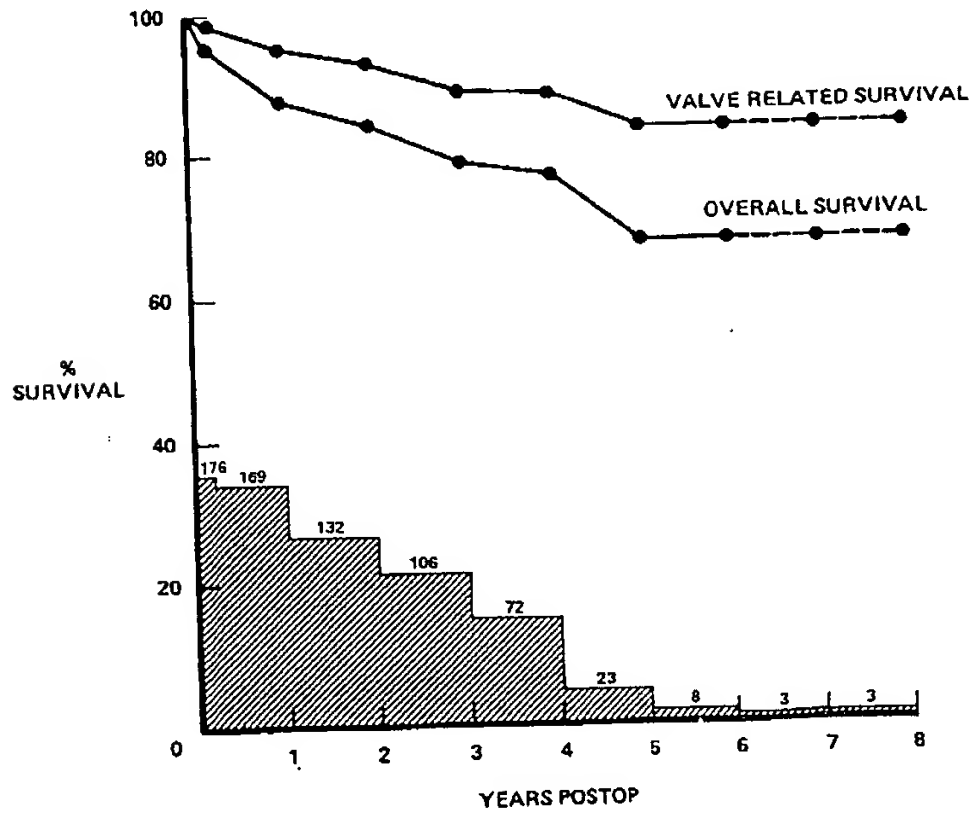
*All overall and valve-related survival curves include operative mortality.

*Table 3. Linearized Mortality and Morbidity Rates*[a]

| | Aortic Valve | Mitral Valve | Multiple Valve |
|---|---|---|---|
| Late mortality | 6.5 | 8.1 | 14.6 |
| Thromboembolism | | | |
| Total | 0.7 | 1.8 | 2.9 |
| Fatal | 0 | 0 | 1.5 |
| Hemorrhage | | | |
| Total | 1.0 | 1.4 | 0 |
| Fatal | 0 | 0 | 0 |
| Endocarditis | | | |
| Total | 0.5 | 0.5 | 4.4 |
| Fatal | 0.2 | 0.5 | 4.4 |
| Tissue failure | | | |
| Total | 0.7 | 0 | 0 |
| Fatal | 0.5 | 0 | 0 |
| Perivalvular leak | | | |
| Total | 3.1 | 0.9 | 5.8 |
| Fatal | 1.0 | 0 | 2.9 |

[a]Values expressed as percent per patient-year.

*Fig 1. Actuarial overall and valve-related 8-year survivals for the 176 patients who underwent aortic valve replacement.*




tuarial valve-related curve* shows 84% survival at 8 years.

The operative mortality for MVR is high at 9%. The linearized late mortality for this group is 8.1% per patient-year with an actuarial overall survival* of 51% at 8 years (Fig 2). However, excluding all deaths known to have been unrelated to the xenograft, the 8-year actuarial valve-related survival* is 94%. Expressed as a linearized rate, the valve-related late mortality is only 0.5% per patient-year.

Although Table 3 shows the statistics for the patients undergoing multiple-valve replacement, these figures do not represent true multiple-valve replacement with the xenograft. Sixty percent of the patients in this group have, in addition to a xenograft, either a mechanical prosthesis or an allograft in place, or they have undergone mitral or tricuspid annuloplasty. For this reason, the valve-related complications or deaths are difficult to determine and, therefore, are hard to analyze. Figure 3 describes the actuarial overall and valve-related survival for this group, and shows, respectively, 48% and 73% of patients alive at 4 years. If operative mortality is excluded, the 4-year overall and valve-related survivals are 63% and 77%, respec-

*All overall and valve-related survival curves include operative mortality.

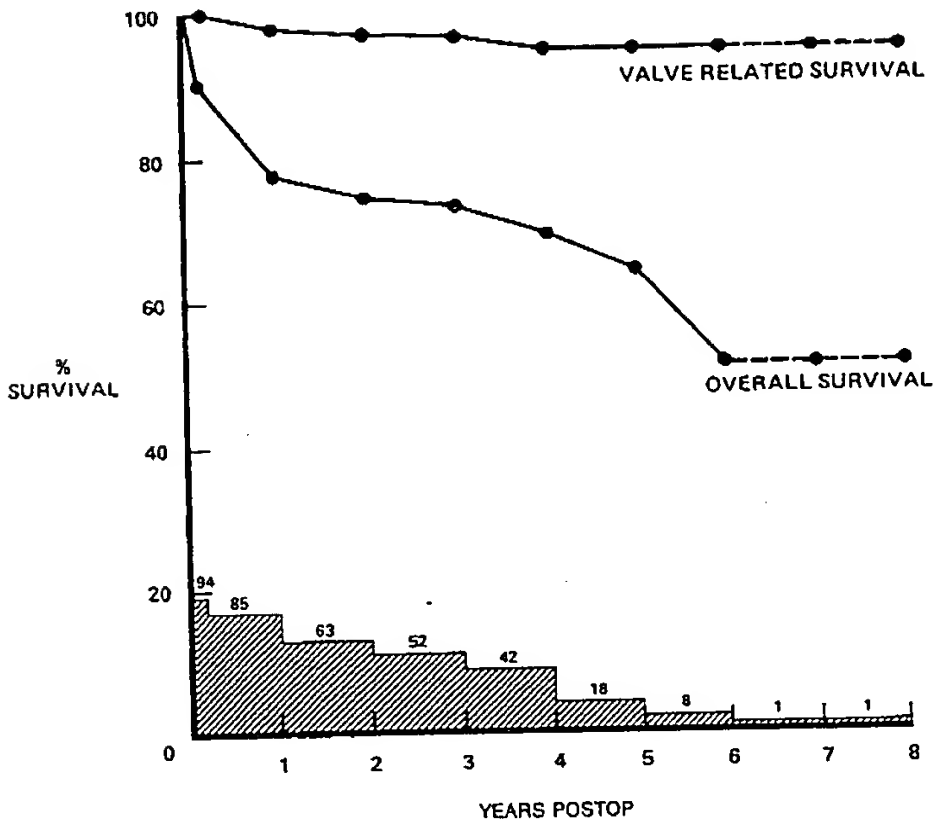



*Fig 2. Actuarial overall and valve-related 8-year survivals for the 94 patients who underwent mitral valve replacement.*

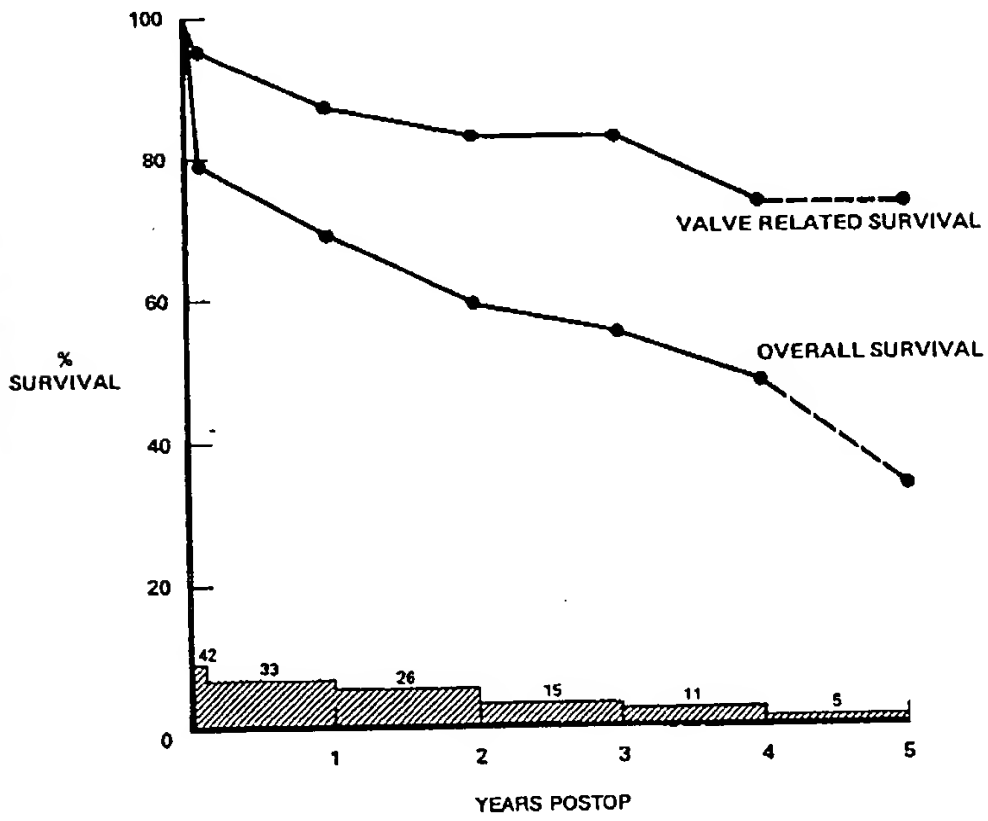


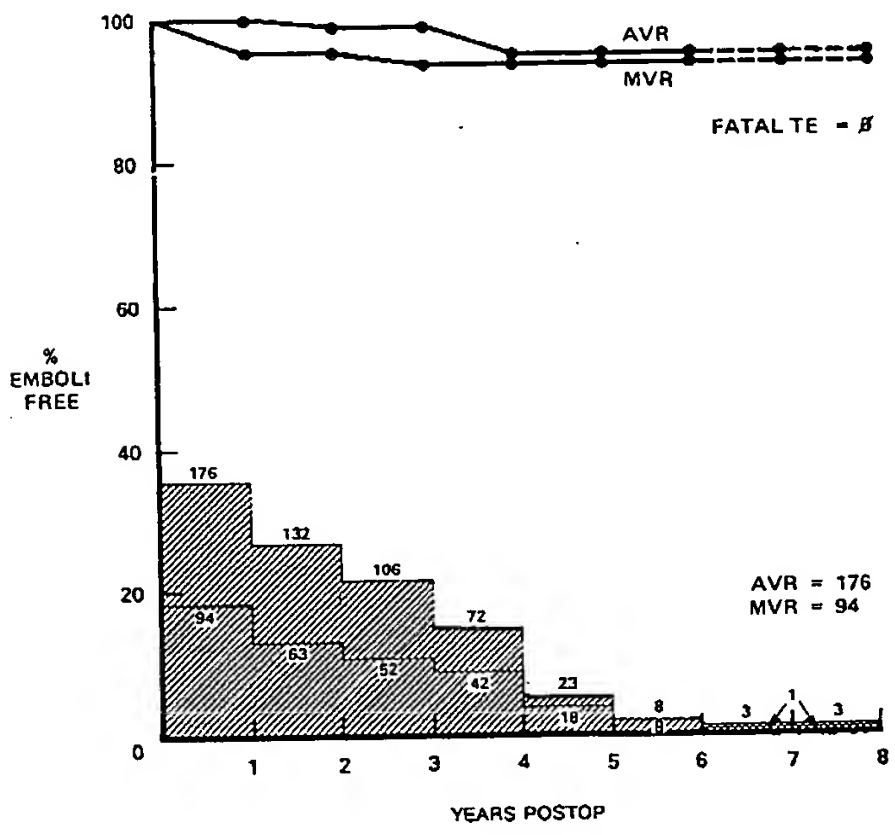

*Fig 3. Actuarial overall and valve-related 8-year survivals for the 42 patients who underwent multiple-valve replacement.*

tively. Expressed as a linearized rate, overall late mortality, including operative deaths, is 14.6% per patient-year. Operative mortality in this group is exceptionally high at 21%. It is significant to note, however, that 56% of the operative deaths (5 of 9) occurred in patients undergoing reoperation (4 patients) or in patients in whom an additional noncardiac procedure was performed (1 patient). Two patients in this group experienced a thromboembolic complication; 1 of them also had an allograft valve in place. Thromboembolism was fatal for 1 patient. Bacterial endocarditis was a fatal complication for 3 patients. In 1 patient, it persisted after removal of an infected allograft, and an allograft valve was present in the second patient dying of endocarditis. Four patients experienced toxic perivalvular leak; it was the cause of death in 2 of them.

*Thromboembolism*

There have been no fatal thromboembolic events in either the AVR or MVR group. The linearized incidence for the AVR group is 0.7% per patient-year, while in the MVR group it is somewhat higher at 1.8% per patient-year. It is significant to note, however, that the rate for patients undergoing MVR is skewed by the occurrence of 75% (3 of 4) of these events during the first 4 postoperative months. Figure 4 expresses the actuarial incidence of thromboembolism in both groups, and shows 95% of the patients who underwent AVR and 94% of those who had MVR to be free from embolus at 8 years.

*Fig 4. Actuarial 8-year incidence of thromboembolism (TE) for aortic and mitral valve replacement.*




*Anticoagulant-related Hemorrhage*

While the risk of anticoagulant-related hemorrhage is expressed as a linearized rate, this value is affected by an uneven distribution of events over the postoperative interval. Five of the 7 hemorrhagic complications occurred within 3 months of operation. As shown in Table 3, the risk for patients undergoing either AVR or MVR is 1% and 1.4% per patient-year, respectively. There were no fatal hemorrhagic events in this series.

*Bacterial Endocarditis*

The linearized occurrence of bacterial endocarditis for both the AVR and MVR groups is identical at 0.5% per patient-year. One of 2 patients who underwent AVR and who had endocarditis died of this complication, resulting in a fatal risk of 0.2% per patient-year. The only

incidence of endocarditis in the MVR group resulted in death.

*Perivalvular Leak*

The risk of developing a perivalvular leak with AVR is 3.1% per patient-year, with an associated linearized mortality rate of 1.0% per patient-year. For patients undergoing MVR, the linearized rate of occurrence is 0.9% per patient-year, with no risk of fatality.

*Tissue Failure*

The occurrence of tissue failure in the AVR group is 0.7% per patient-year, with a linearized mortality rate of 0.5% per patient-year. No tissue failure was experienced in the MVR group.

*Hemodynamic Function*

Two patients (1 each from the AVR and MVR groups) in this series have undergone reoperation for significant transvalvular gradients. The patient requiring AVR, an extremely vigorous 25-year-old man with hyperdynamic left ventricular contractility and a high cardiac output (7.3 liters per minute), was found 13 months postoperatively to have a 51 mm mean gradient across a 24 mm xenograft. At reoperation, the valve was noted to be normally flexible and without calcification. In the second patient, a 19 mm mean gradient was measured across a 31 mm mitral valve xenograft 10 months after implantation. The removed valve demonstrated inherently stiff leaflets without calcific changes.

Clinically significant stenosis is present in 2 patients with small annulus diameter valves in the aortic (23 mm) and mitral (27 mm) position. While both patients are minimally symptomatic 26 and 46 months postoperatively, they may well require reoperation.

Three patients who underwent AVR have clinically insignificant murmurs of xenograft insufficiency, one of which is thought to be perivalvular in origin. This condition has been stable in all 3 patients for 14, 18, and 30 months, respectively.

Examination of two valve specimens from patients dying of left ventricular failure in a low output state, showed fibrin deposition in the smallest cusp of these large, asymmetric valves.

**Comment**

The hospital thirty-day survival rates for both the AVR and MVR groups are not enviable, and in part, are explained by the exclusive use of the porcine xenograft during the past 5 years (1973 through 1978). As in any consecutive series, this early mortality is more often a reflection of the patient's preoperative age and New York Heart Association Functional Class than of the valve's performance. This is borne out by the valve-related survival and complication curves. Table 4 lists the causes of hospital death in both patient groups; only 1 patient, who was undergoing AVR, died of causes related to the valve. In this patient, acute dehiscence of the valve resulted in cardiovascular collapse. In 3 patients with AVR, sepsis was an important factor in their deaths. In 1 of them with a sternal infection, a persistent, resistant bacteremia de-

*Table 4. Causes of Operative Death in Two Groups of Patients Undergoing Aortic or Mitral Valve Replacement*

| Valve[a] | Cause of Death[b] |
|---|---|
| Aortic (4%) | Intraoperative CVA (67) |
| | Sudden, unexplained (96) |
| | Bleeding (162) |
| | Reoperation for dehisced xenograft (183) |
| | Sternal infection (236) |
| | Renal failure and sepsis (256) |
| | Pulmonary infection (314) |
| Mitral (9%) | MI, s/p S.E. valve for TE, MI (40) |
| | Low output, TVD, ↓LV function (64) |
| | Aspiration, tracheostomy, TVD (197) |
| | Hyperkalemic arrest, RP clot (212) |
| | Renal failure, TVD, ↓ LV function (223) |
| | Shock, ventilator, ↑ Syst PAP (231) |
| | PI and fibrosis, cachectic (300) |
| | Low output, ↑ Syst PAP, TVD (330) |

[a]Percentage represents hospital mortality.
[b]Number in parentheses is that of the glutaraldehyde-treated porcine xenograft involved. Total number inserted = 331.

CVA = cerebrovascular accident; TVD = triple-vessel disease; LV = left ventricular; MI = myocardial infarction; s/p = status post; S.E. = Starr-Edwards; TE = thromboembolism; RP = retroperitoneal; Syst PAP = systemic pulmonary artery pressure.

veloped and was the cause of death. Preoperative long-term steroid therapy for control of obstructive pulmonary disease contributed to pulmonary infection and death in a second patient. A 78-year-old man died of sepsis resulting from bowel perforation during peritoneal dialysis for renal failure. The remaining operative deaths in the AVR group were due to an intraoperative cerebrovascular accident and to complications secondary to persistent bleeding; a sudden, unexplained death 1 week following discharge was presumed to have been due to an arrhythmia.

Five of the 9 hospital deaths in the MVR group were the result of complications secondary to poor left ventricular function and concomitant triple-vessel coronary artery disease. A cachectic patient with pulmonary fibrosis and insufficiency and in whom mitral valve regurgitation was of questionable significance, continued to deteriorate postoperatively and eventually died. One patient died of cardiac arrest secondary to hyperkalemia, which was probably aggravated by an old retroperitoneal hematoma. One patient with a mechanical prosthesis died of a myocardial infarction and pulmonary insufficiency during reoperation. Interestingly, this patient is the only hospital death among the 24 patients with isolated MVR who underwent reoperation.

The first year mortality was also high in both the AVR (13 patients) and MVR (10 patients) groups. With MVR, only 2 of the deaths were proved to be valve-related. One patient died of persistent bacterial endocarditis, and 1 died suddenly of an unexplained cause presumed to be valve-related. With AVR, 7 patients died of proved or possible valve-related causes within the first postoperative year. Three of these deaths were secondary to complications of perivalvular leak, 1 was due to bacterial endocarditis, and 3 were sudden, unexplained, and presumed valve-related. For the remaining 14 patients who died (8, MVR; 6, AVR), it was known that the cause of death was not valve-related or else at postmortem examination the xenograft was shown to be normal or at catheterization before death it was functioning normally. Table 5 lists the non-valve-related 1-year mortality in both patient groups.

*Table 5. Non-Valve-Related Deaths in Postoperative Year 1*

| Valve | Cause of Death[a] |
|---|---|
| Aortic | Pulmonary embolus (12) |
| | Arrhythmia (137) |
| | Arrhythmia (144) |
| | Suicide (169) |
| | LV failure (274) |
| | Hepatitis (225) |
| Mitral | Suicide (31) |
| | Arrhythmia (33) |
| | LV failure (80) |
| | Cirrhosis of liver (114) |
| | LV failure (189) |
| | LV failure (249) |
| | Pneumonia (312) |
| | LV failure (307) |

[a]Number in parentheses after cause of death is that of the glutaraldehyde-treated porcine xenograft involved. Total number inserted = 331.

LV = left ventricular.

In the AVR group, valve-related mortality in years 2 through 8 was the result of tissue failure by calcific stenosis in 2 patients. Five patients died suddenly of unknown cause, which was presumed valve-related. The remaining 7 patients died of causes clearly not related to the xenograft. None of the late deaths occurring in the MVR group were proved to be valve-related. One patient died of an unexplained, presumed valve-related cause, and the remaining 8 patients were shown to have died of unrelated causes or to have normal xenografts postmortem or at catheterization. Table 6 describes the causes of late death in both the AVR and MVR patient groups.

A group of 42 patients includes those who underwent reoperation for a previous aortic or mitral valve surgical procedure. There were 2 operative deaths, for a hospital mortality of 4.8%. This is less than that for patients undergoing isolated valve replacement for the first time. The low surgical mortality in this group, the majority of whom were having reoperation because of allograft or formalin xenograft failure, is a strong point in favor of the use of tissue valves, indicating a low risk to the patient should replacement due to valve

*Table 6. Cause of Late Deaths in Postoperative Years 2 through 8*[a]

| Valve | Cause of Death: Non-Valve-Related | Cause of Death: Valve-Related |
|---|---|---|
| Mitral | CAD and hepatitis (109) | Sudden, unexplained (14) |
| | Cancer (213) | |
| | LV failure (191) | |
| | Operation for other valve disease (115) | |
| | Cancer (119) | |
| | CHF (5) | |
| | Other valve disease (28) | |
| Aortic | Perforated peptic ulcer (83) | Sudden, unexplained (55) |
| | Liver failure (103) | Sudden, unexplained (143) |
| | Cancer (182) | Sudden, unexplained (139) |
| | MI with TVD (74) | Sudden, unexplained (153) |
| | CHF (99) | Sudden, unexplained (195) |
| | LV failure (78) | Xenograft stenosis (16) |
| | Cancer (2) | Xenograft stenosis (20) |

[a]Number in parentheses after cause of death is that of the glutaraldehyde-treated porcine xenograft involved. Total number inserted = 331.

CAD = coronary artery disease; LV = left ventricular; CHF = congestive heart failure; MI = mitral insufficiency; TVD = triple-vessel disease.

failure be necessary. Two patients in the present xenograft series failed to return for reoperation necessitated by marked calcific aortic stenosis, and both died. This points out the importance of close patient follow-up with postoperative catheterization or other definitive evaluation at the first indication of possible valve failure.

No patient in this series sustained a fatal thromboembolism. However, as reported here and elsewhere [9, 13, 21],* the early risk of this event, particularly with MVR is significant, and supports the concept of an early anticoagulation regimen. It is important to note that two of the three early thromboembolic events in the MVR group (Table 7) coincided with the abrupt cessation of anticoagulation therapy due to hemorrhagic complication. Late thromboembolism in both groups (4 patients) can only be presumed valve-related and may, in fact, be noncardiac in origin. The actuarial occurrence of thromboembolic events during an 8-year interval is shown in Figure 4.

The incidence of anticoagulant-related hemorrhage is in agreement with that reported by

*Oyer PE: Personal communication, 1979.

*Table 7. Occurrence of Thromboembolism and Hemorrhage by Postoperative Interval*

| Interval (mo) | AVR | MVR |
|---|---|---|
| THROMBOEMBOLISM | | |
| 0–12 | None | Stroke: hom hem |
| | | Stroke: memory loss |
| | | Stroke: no deficit |
| 12–24 | TIA—APHASIA | None |
| 24–36 | None | Stroke: no deficit |
| 36–48 | TIA—APHASIA | None |
| | TIA—APHASIA | |
| HEMORRHAGE | | |
| 0–12 | GI bleed | Subdural hematoma |
| | GI bleed | Hematuria |
| | Vaginal bleed | GI bleed |
| 12–24 | GI bleed | None |
| 24–36 | None | None |
| 36–48 | None | None |

AVR = aortic valve replacement; MVR = mitral valve replacement; hom hem = homonymous hemianopia; TIA = transient ischemic attack; GI = gastrointestinal.

other investigators [9, 22]: While low, it remains a disturbing complication, occurring during short-term therapy in 3 of our patients who had AVR and 3 who underwent MVR, and in 1 patient who had MVR and 1 who had AVR who were on a long-term regimen of anticoagulation. Further reduction of this incidence may be possible only by more rigorous control or by accepting a shorter prothrombin time. Most important is the observation that in no patient was hemorrhage fatal.

Bacterial endocarditis, excluding patients who had more than one type of valve prosthesis in place, occurred at a rate of 0.4% per patient-year for the combined MVR (1 patient) and AVR (2 patients) groups. Two patients died as a result of this complication, 1 who had undergone replacement with a xenograft due to infection of the previously placed autograft. One patient was treated successfully.

Perivalvular leak is the only serious valve-related complication encountered in this 8-year experience. This problem, which was seen to occur secondary to annular disruption from toxic glutaraldehyde present in the sewing flange at implantation, reached disastrous proportions in the fifth year (1975) of our series. In a 1-month period, 43% of 14 patients operated on for valve replacement experienced this complication. Careful retrospective analysis of our own early experience showed a rather persistent, and for us, unusual occurrence of perivalvular leak in the absence of any predilection on the part of the patient. Furthermore, at the time of reoperation, we observed poor tissue ingrowth and suture distraction even in a valve recipient with a healthy annulus. While an extensive search of the literature failed to reveal any series reporting a similar problem, direct communication with other users of the glutaraldehyde xenograft disclosed similar, unexplained incidences of lesser magnitude [9, 10, 23]. The presence of annular necrotic tissue loss, microaneurysm formation (Fig 5), and abscess in the absence of infection was thought to implicate a toxic, noninfective process with no apparent relationship to operative technique or valve construction. In vivo and in vitro testing for toxic substances in the plastics, fabrics, or solutions used in valve fabrication failed to identify any agent other than glutaraldehyde. Further analysis of the valve storage solutions revealed the presence of high concentrations of long-chain glutaraldehyde polymers known to be poorly soluble in aqueous solution. Subcutaneous implantation in rabbits of fabric samples stored in these insoluble, high-polymer content solutions was seen to produce abscess formation. Purification of glutaraldehyde solutions with further control of glutaraldehyde polymer content by strict adherence to recommended storage conditions (temperatures of 4° to 18°C and avoidance of exposure to ultraviolet light) and the use of a rigorous rinse protocol prior to implantation have been effective in eliminating perivalvular leak from this series.

*Fig 5. Microaneurysm formation resulting from glutaraldehyde toxicity.*

Since October, 1975, 135 glutaraldehyde xenografts have been implanted without incidence of perivalvular leak. Valves retrieved postmortem have shown good healing in the perivalvular region without microscopic evidence of tissue disruption or inflammatory reaction. It is believed that the cause of this problem has been identified and corrected. However, we believe that information regarding the significance and potential for occurrence of this complication with all types of glutaraldehyde xenografts has not been appropriately disseminated. Other groups using the glutaraldehyde xenograft have experienced un-

explained perivalvular leak, and we think an even higher occurrence may be obscured by bacterial endocarditis occurring secondary to toxic perivalvular disruption.

Tissue failure is rare in this series and was observed only in 3 patients undergoing AVR. Calcification with resultant stenosis was the cause of death in 2 patients at 2.5 and 4.5 years postoperatively. The histological changes observed in these valves are of great concern. Ongoing study of long-term valves indicates that in some there is a gradual loss of architectural integrity with the insinuation of blood and serum into the valve substance, slow lamination of the cusp, and subsequent calcification of the lesion. This process of calcification seems to occur with a strikingly increased frequency in children and in patients with aberrant calcium metabolism, such as those in renal failure [7, 12, 18, 25].* Cusp perforation was seen in 1 patient reoperated on at 6 months for aortic insufficiency. Subsequent shrink temperature testing of the valve leaflets revealed incomplete glutaraldehyde cross-linking. While tissue disruption has been reported in other series with the Hancock valve, the incidence, at present, is not significant [7, 9, 12–14, 20, 22].

*Hemodynamic Performance*

The hemodynamic evaluation of valve replacement devices is a complex task. While there is excellent correlation between flow and gradients under steady-state in vitro conditions, marked disparity is reported between the in vitro and in vivo data [2, 15, 17, 19, 21, 26, 27]. These correlations are complicated further by the normal physiological variations that occur particularly during exercise. The left ventricular dynamics, cardiac chamber size, and heart rate together affect the peak flow rate and the shape of the pulsatile flow curve so profoundly that, in individual patients, valve size is a relatively poor predictor of measured in vivo gradients.

In our experience, the aortic xenograft behaves as a fixed orifice valve. The steady-state flow curve is seen to be a straight line function on a log-log graph, with a plus or minus 10% standard deviation among valves of the same implantation diameter. Figure 6 illustrates how the in vivo gradients measured across a 24 mm valve in the aortic position of 10 patients compare with the in vitro steady-state flow curve. Peak flow rates are seen to vary from 6 to 23 liters per minute (330%), and the measured gradients fluctuate fourfold from 6 to 24 mm Hg. The average peak flow rate and valve gradient of these 10 patients are 13.4 liters per minute and 19.0 mm Hg, respectively.

The aortic valve gradients measured intraoperatively (51 patients) and at postoperative catheterization (18 patients) are shown in Figure 7. There is considerable scatter of these data points, as would be expected, because of some variation in flow orifice among valves of the same annulus diameter and because of the physiological variability in peak flow from patient to patient. In spite of this scatter, however, there is a general decrease in the measured gradient from the 21 mm to the 30 mm valves in the aortic position. Furthermore, as shown in Table 8, averaging the in vivo (intraoperative and catheterization) gradients measured in valves 24 mm and smaller, there is a correlation between this value (19.2 mm Hg) and the predicted steady-state gradient of 19 mm Hg assuming a peak flow of 13.4 liters per minute (cardiac output = 4.4 liters per minute). For aortic valves 25 mm and larger, the 13.5 mm Hg average of intraoperative (13.8 mm Hg) and catheterization (11.3 mm Hg) gradients correlates with the in vitro steady-state value of 12 mm Hg assuming a peak flow of 15 liters per minute (cardiac output = 5.2 liters per minute).

For valves in the mitral position, most reported hemodynamic data attempt to correlate valve size with measured diastolic mean gradients and calculated valve orifice areas. As with the aortic xenograft, in vitro steady-state gradients are consistent, straight line, log-log functions except at low flow rates and low pressure gradients. Under these latter conditions, leaflet flexibility becomes a significant factor in valve hemodynamics. If the cardiac output is measured at less than 2 to 2.5 liters per minute and if the change in pressure is less than 2 mm Hg, our studies as well as those of Thompson and Barratt-Boyes [26] and Wright [27] show

*Oyer PE: Personal communication, 1979.

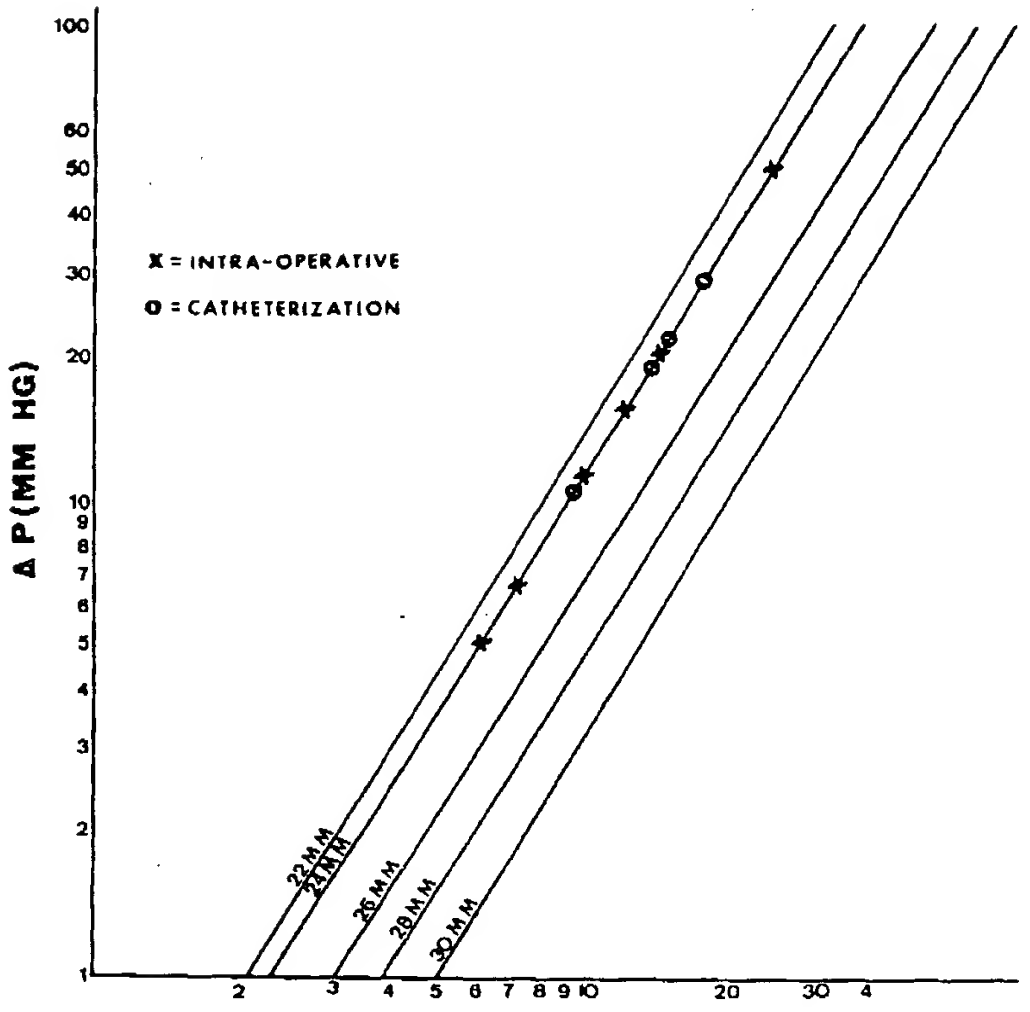



*Fig 6. A comparison between in vivo aortic valve gradients measured with a 24 mm Angell-Shiley xenograft and the in vitro steady-state flow curve.*

that the valve leaflets only open partially and that the third valve leaflet, usually the right coronary cusp, moves poorly. Wright's study of 5 tissue valve types also indicates that in the mitral position, the third leaflet is, on the average, not fully open until a flow of 12 liters per minute is achieved (cardiac output = 5 liters per minute). Of the valves tested by Wright, initial opening of the third leaflet is seen to occur at flows as low as 1.5 liters and, in some cases, this leaflet does not fully open until a flow of 18 liters is attained. While Wright does show some correlation of this behavior with valve type, there is more variability among valves of the same type than between kinds of valves. For MVR, this inherent leaflet inflexibility most likely accounts for the poor correlation reported between the valve orifice size and the measured in vivo gradient [15, 19].

In spite of these variables, in our present series, the average measured in vivo gradient correlated with valve size in the mitral position. As noted in Table 9, the average intraoperative and catheterization gradient for 26 to 29 mm valves was 5.3 mm Hg, and for 30 to 33 mm valves the average value was 2.4 mm Hg. This agrees reasonably well with the in vitro data of Wright [27] if one assumes that a mean flow of 140 ml per second is equivalent to a cardiac output of 4.4 liters per minute. Under those conditions, Wright measured a 6 mm Hg gradient in the 27 to 29 mm sizes and a 4 mm Hg gradient in the 30 to 34 mm sizes of the Angell-Shiley valve. In addition, our own steady-state in vitro data would predict 4.1 and 2.3 mm Hg gradients for the smaller and larger valves, respectively, assuming a cardiac output of 4.4 liters per minute, with a peak flow of 8.8 liters per minute.

From a clinical standpoint, although the effective flow orifice is less than optimal, the xenograft has permitted adequate function in 98.6% (283 of 287) of surviving patients. In our experience, 1 aortic and 1 mitral valve have required removal because of a high gradient. The

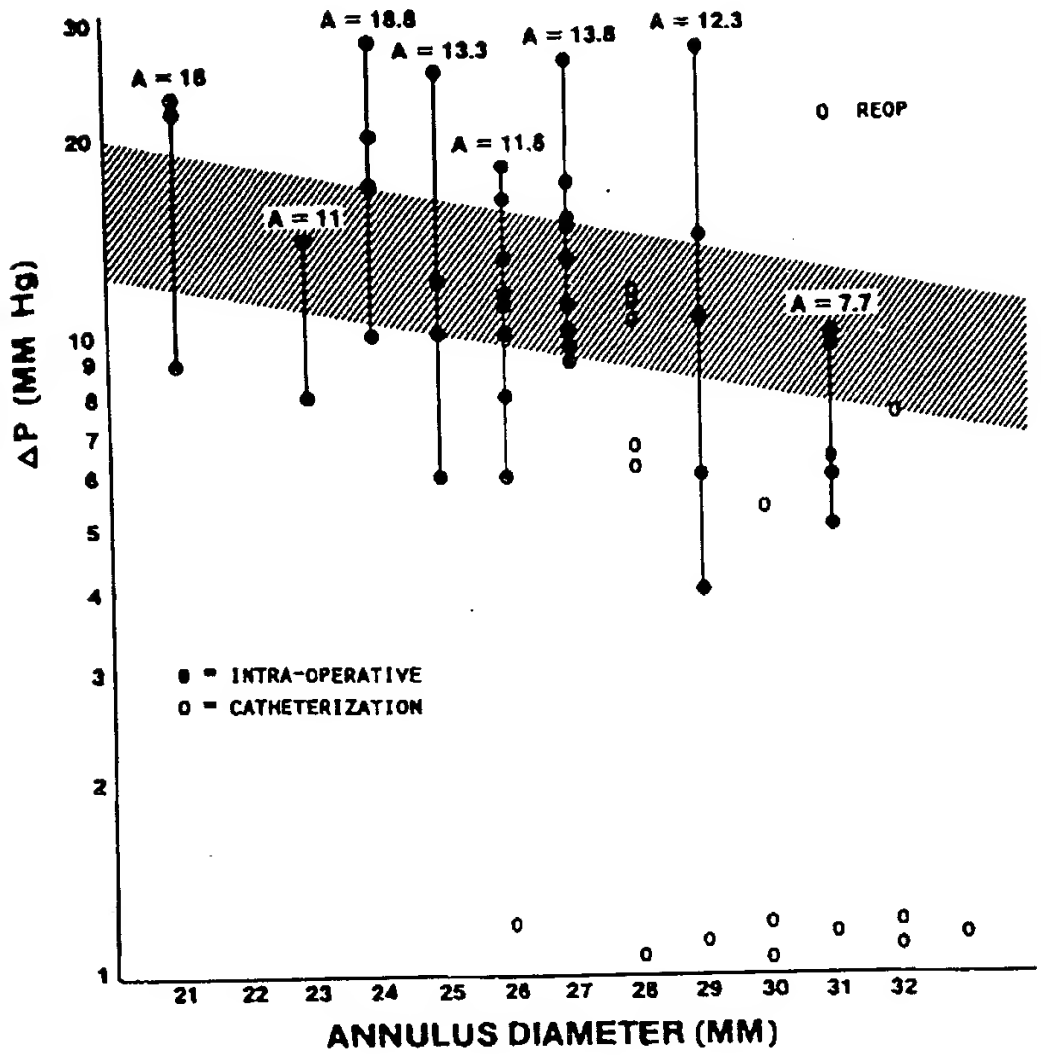



*Fig 7. In vivo gradients measured intraoperatively and at postoperative catheterization with an Angell-Shiley xenograft aortic valve replacement.*

*Table 8. Hemodynamic Measurements for Valves in the Aortic Position*

| Gradient (mm Hg) | Annulus Diameter 24 mm or less | Average | Annulus Diameter 25 mm or more | Average |
|---|---|---|---|---|
| Intraoperative | 18.2 (19 pts) | 19.2 | 13.8 (42 pts) | 13.5 |
| Catheterization | 20.8[a] (12 pts) | | 11.3[b] (6 pts) | |
| In vitro steady-state | 19.0[a] | (19.0) | 12.0[b] | (12.0) |

[a]At a cardiac output of 4.4 liters per minute.
[b]At a cardiac output of 5.2 liters per minute.
pts = patients.

*Table 9. Hemodynamic Measurements for Valves in the Mitral Position*

| Gradient (mm Hg) | Annulus Diameter 29 mm or less | Average | Annulus Diameter 30 mm or more | Average |
|---|---|---|---|---|
| Intraoperative | 4.3 (15 pts) | 5.3 | 2.5 (15 pts) | 2.4 |
| Catheterization | 7.5[a] (7 pts) | | 2.2[a] (9 pts) | |
| In vitro steady state | 4.1[a] | (4.1) | 2.3[a] | (2.3) |

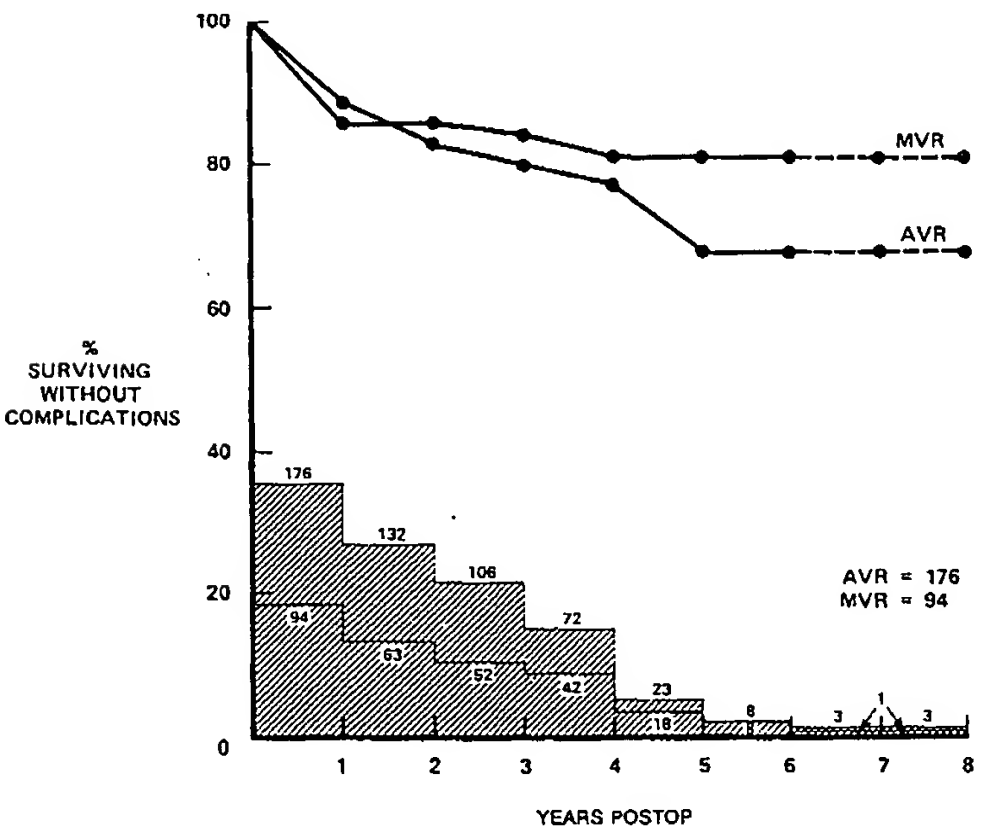
[a]At a cardiac output of 4.4 liters per minute.
pts = patients.

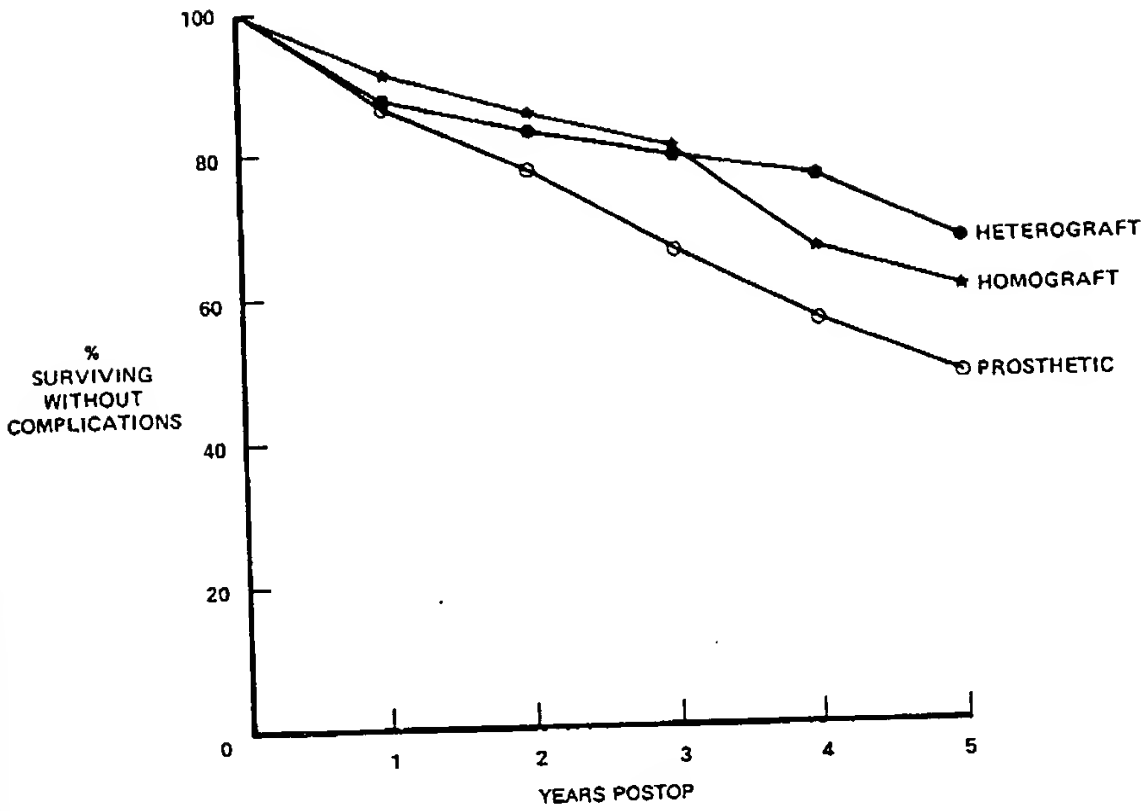
23 mm aortic xenograft did not provide an adequate flow orifice for a vigorous 25-year-old man with a high cardiac output and a hyperdynamic left ventricular contractility. Reoperation included considerable patch widening of the aortic root to enable placement of a larger 28 mm valve. The 30 mm mitral xenograft that was removed demonstrated inherently stiff leaflets without calcification; this resulted in a high gradient despite adequate size. Two additional patients, 1 with a 23 mm aortic xenograft and 1 with a 27 mm mitral xenograft, have hemodynamically significant gradients at postoperative catheterization. These patients may well need reoperation, although at present they are minimally symptomatic.

As mentioned earlier, poor leaflet mobility in the low cardiac output state (less than 2.5 liters per minute) is well demonstrated by cinephotography. In our series, 2 patients dying of non-valve-related cause in a low output state were found at postmortem examination to have fibrin deposition behind the smallest leaflet of the large asymmetric valve. It is believed that the combination of a large valve in a patient with an initially low cardiac output may result in fibrin deposition and cause a progressively higher valve gradient as the cardiac output increases in the later postoperative course. These observations have prompted us to maintain a high cardiac output in the early postoperative period and to initiate early anticoagulation treatment in order to reduce the deposition of fibrin and platelets around the functional portions of the valve leaflets.

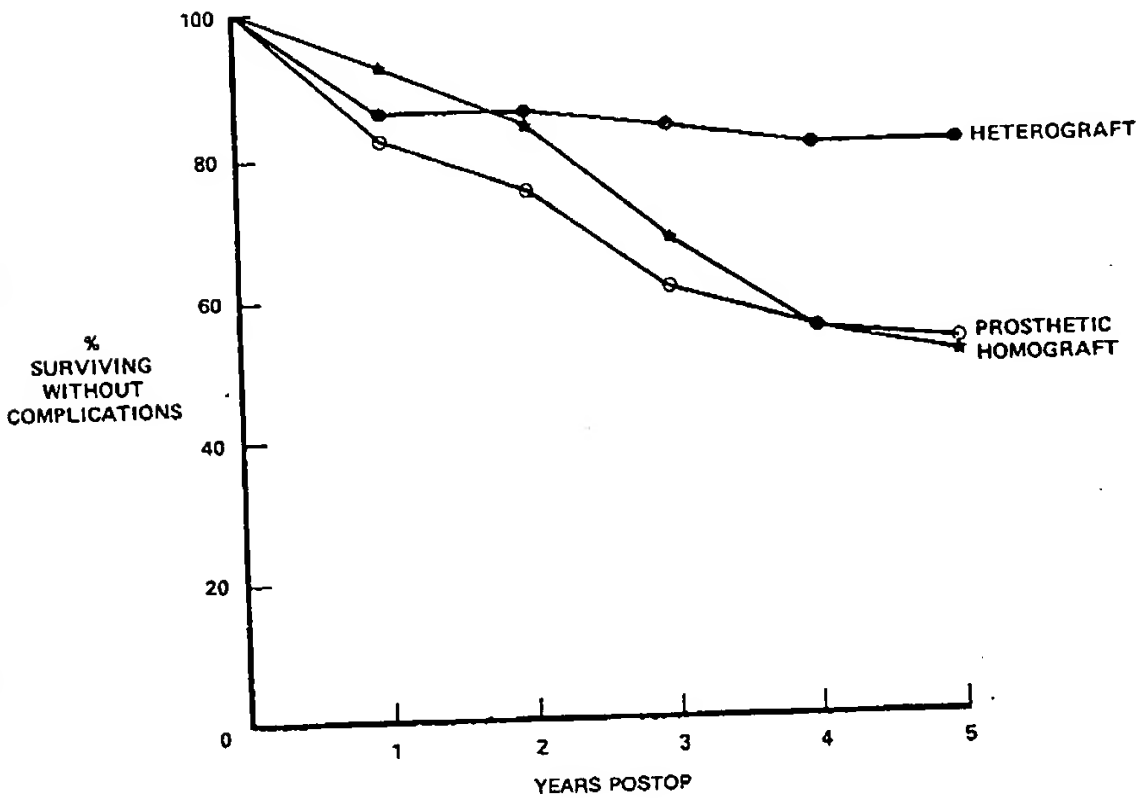
By comparison with the mechanical prostheses, tissue valves such as the allograft and the glutaraldehyde xenograft are seen to have higher transvalvular gradients, both in vivo and in vitro. As indicated by the study of Wright [27], some hemodynamic differences exist between the Angell-Shiley, Hancock, and Carpentier-Edwards valves, but there is clearly more variation in measured hemodynamic data from patient to patient and valve to valve than is reported by the three manufacturers [2]. Newer tissue valve types such as the Ionescu-Shiley [27], the Hancock 250 series [17], and the prototype high-flow orifice Angell-Shiley valve are examples of the fact that most of the hemodynamic disadvantages seen with the tissue valve are solvable problems. The annulus to flow orifice ratio of the glutaraldehyde-treated tissue valve can be im-

*Fig 8. Valve-related morbidity and mortality among 270 patients who underwent aortic or mitral valve replacement.*






A




B

*Fig 9. Comparison of actuarial 5-year morbidity and mortality among xenograft, allograft, and mechanical prosthesis for (A) patients who had aortic valve replacement and (B) patients who had mitral valve replacement.*

proved to such a point that it is comparable to the best mechanical prosthesis. It is essential, however, that the proved intrinsic advantages of the xenograft valve not be compromised in these alterations.

## Conclusion

In our opinion, the most meaningful analysis of xenograft function is represented by actuarially determined valve-related morbidity and mortality. As shown in Figure 8, 81% of the patients who underwent MVR and 68% of the patients who had AVR have survived 8 years without experiencing a valve-related complication. The 13 patients (7.5% with AVR) who sustained toxic perivalvular leak during the first

postoperative year account for 50% (13 of 26) of the total number of valve-related complications and deaths in this group. Fortunately, the problem has been identified and has not recurred in the last 3 years.

A comparison of AVR and MVR with the xenograft, mechanical prostheses and the fresh allograft over a similar 5-year follow-up is noted in Figure 9. While retrospective studies defy statistically significant correlation, we believe, on the basis of past experience, that these trends and relationships are meaningful indicators of relative valve performance and risk to the patient. The comparison for patients undergoing AVR shows results with the xenograft to be superior to those with mechanical prostheses and not significantly different from those with the allograft. For MVR, the xenograft is seen to offer a significantly reduced risk of valve-related death and complication over either the allograft or the mechanical prostheses.

In this experience, thromboembolism and anticoagulant-induced hemorrhage are reduced to a minimum and a nonfatal incidence of 1.1% per patient-year. Valve failure is insignificant in the first 8 years of follow-up (0.5% per patient-year), and hemodynamic performance has been acceptable in all but 1.6% (4) of the surviving patients. We anticipate that improved stent design and glutaraldehyde fixation methods will further enhance valve hemodynamics and long-term function.

We continue to use the Angell-Shiley xenograft as the valve of choice for isolated aortic, mitral, and tricuspid replacement as well as for combined and complex procedures.

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## Notice from the American Board of Thoracic Surgery

The American Board of Thoracic Surgery now requires that candidates pass both the written and oral portions of the certifying examination.

In 1980 and thereafter, a written examination will be given *prior* to the oral examination. It will be necessary to pass the written examination before the oral examination can be taken. The closing date for registration for 1981 is August 1, 1980. The exact times and places of these examinations will be announced later.

A candidate applying for admission to the 1981 certifying examination must fulfill all the requirements for the Board in force at the time the application is received.

Please address all communications to the American Board of Thoracic Surgery, 14640 E Seven Mile Road, Detroit, MI 48205.

# Delayed rejection of porcine cartilage is averted by transgenic expression of α1,2-fucosyltransferase[1]

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## SPECIFIC AIMS

In our laboratory we aim to develop porcine cartilage resistant to delayed tissue rejection for xenotransplant applications. First it was necessary to understand the process of xenogeneic cartilage rejection, particularly the contribution of the Galα1,3-Gal antigen present in the donor tissue. To this end, in vitro engineered porcine cartilage expressing α1,2-fucosyltransferase (HT) was generated and tested in vivo in wild-type and α1,3-galactosyltransferase knockout (Gal KO) mice.

## PRINCIPAL FINDINGS

### 1. Transgenic expression of HT reduces dramatically the amount of Galα1,3-Gal antigen in porcine cartilage and isolated chondrocytes

We characterized porcine articular chondrocytes (PAC) isolated from control and two lines of HT transgenic pigs ($HT^{AT20}$ and $HT^{AT21}$) by flow cytometry. $HT^{AT20}$-expressing cells showed the most reduction (89%) in Galα1,3-Gal epitope cell surface expression as well as diminished human and Gal KO-mouse natural antibody reactivity. The pattern and level of Galα1,3-Gal expression were investigated in control and $HT^{AT20}$ transgenic cartilage by immunofluorescence. A generalized decrease in Galα1,3-Gal antigen was detected in both native and in vitro engineered HT transgenic cartilage.

### 2. $CD4^+$ T cells play a major role in xenogeneic porcine cartilage rejection by inducing an anti-pig antibody response and a monocytic cellular infiltrate

To characterize the process of xenogeneic cartilage rejection in a small animal model, we first studied the contribution of $CD4^+$ T cells to the rejection of $HT^{AT21}$ porcine cartilage implanted subcutaneously (s.c.) in wild-type mice. The humoral response was evaluated by assessing the anti-PAC antibody reactivity in serum at 3 and 5 wk after transplantation. Treatment with a depleting anti-CD4 antibody completely blocked the elicited antibody response, which was predominantly anti-pig and showed no specific reactivity to the H epitope. The effect of blocking $CD4^+$ T cells on the rejection process was also analyzed by histology. Implants from the control-treatment group showed a predominantly mononuclear cellular infiltrate surrounding the xenograft at 3 and 5 wk. Treatment with anti-CD4-depleting antibody markedly reduced the amount of cellular immune infiltrate at both time points, but a minor lymphocytic infiltrate remained in the implant periphery.

### 3. HT porcine cartilage is highly resistant to delayed tissue rejection in Gal KO mice

To test the effect of reducing Galα1,3-Gal antigen in the donor tissue, we s.c. transplanted control or $HT^{AT20}$ engineered cartilage into Gal KO mice. Histological evaluation showed that the nontransgenic porcine cartilage was rejected as early as 2.5 wk, with extensive tissue destruction and a pronounced mononuclear cellular infiltrate penetrating the tissue (**Fig. 1*A***). The cellular infiltrate progressed and peaked at 5 wk (Fig. 1*C*). This rejection process was faster and more aggressive than that observed in grafted wild-type mice, but similarly contained cells from monocyte/macrophage and lymphocyte lineages. In contrast, the $HT^{AT20}$ transgenic grafts were intact at 2.5 wk (Fig. 1*B*) and the cartilage structure was preserved during the course of the study (5 and 10 wk) (Fig. 1*D*, *F*). Rejection was limited to the xenograft periphery, where a minor cellular infiltrate developed.

### 4. The elicited antibody response is markedly reduced in Gal KO mice transplanted with HT cartilage grafts

To investigate the mechanism of Galα1,3-Gal-mediated rejection, we assessed the humoral response in the two transplanted cohorts (**Fig. 2**). Basal levels of anti-PAC IgM antibody reactivity were detected in nontransplanted Gal KO mice by flow cytometry. The anti-PAC

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02–0630fje; to cite this article, use *FASEB J.* (November 15, 2002) 10.1096/fj.02–0630fje

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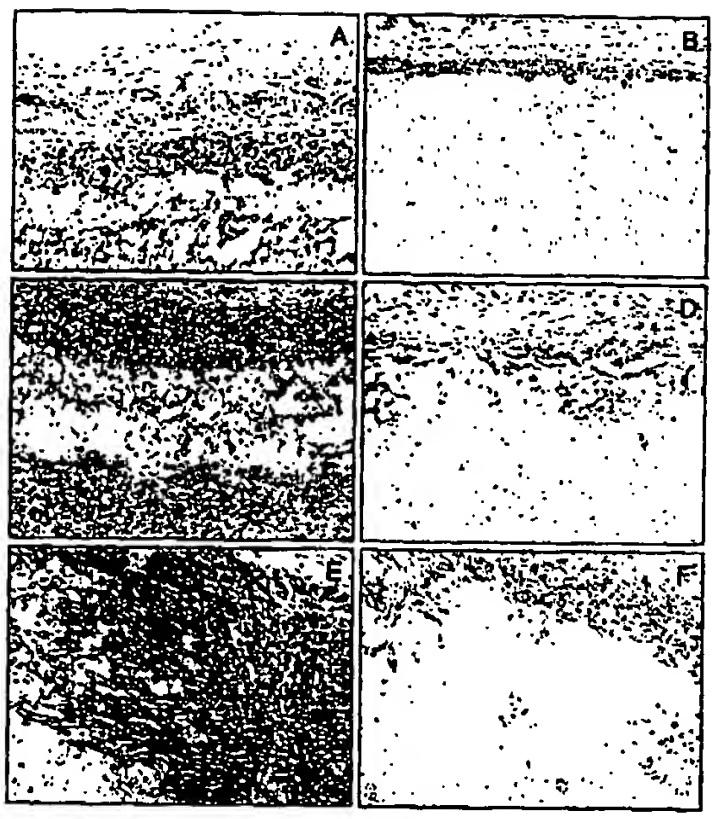


Figure 1. Histological analysis of control and $HT^{AT20}$ engineered cartilage implanted s.c. in Gal KO mice. Hematoxylin and eosin staining of the following sections is shown: control engineered cartilage harvested at 2.5 wk (*A*), 5 wk (*C*), and 10 wk (*E*) post-transplantation, as well as HT engineered cartilage harvested at 2.5 wk (*B*), 5 wk (*D*), and 10 wk (*F*) post-transplantation.

IgM antibody titers rose at 5 wk post-transplantation in the group receiving control cartilage grafts and remained high up 10 wk (Fig. 2*A*). In contrast, Gal KO mice receiving $HT^{AT20}$ cartilage grafts showed no elevation in anti-PAC IgM antibodies above background (Fig. 2*A*). Consistent with a predominantly anti-Galα1,3-Gal IgM response in the control graft recipients, reactivity of the IgM subtype to HT transgenic PAC was comparable between experimental groups and was overall lower than to control PAC (Fig. 2*C*). With regard to anti-PAC IgG antibodies, no reactivity was detected in serum from nontransplanted Gal KO mice (Fig. 2*B, D*). Mice receiving control cartilage mounted an anti-PAC IgG response first detected 2.5 wk after transplantation, peaking at 5 wk (Fig. 2*B*). $HT^{AT20}$ graft recipients showed no detectable anti-PAC IgG at 2.5 wk, lower anti-control-PAC humoral response at 5 wk (2-fold reduction vs. control transplant group), and near-background IgG reactivity at 10 wk (Fig. 2*B*). Results with $HT^{AT20}$ transgenic PAC suggested that the increase in IgG antibody titers in response to the $HT^{AT20}$ cartilage grafts was predominantly non-anti-Galα1,3-Gal (Fig. 2*D*). We later confirmed by ELISA that the elicited anti-Galα1,3-Gal antibody response was absent in Gal KO mice grafted with HT cartilage. Moreover, we assayed splenic B cells for reactivity to the Galα1,3-Gal epitope by flow cytometry. Whereas mice receiving control cartilage showed an increase in the percentage of B cells binding to the Galα1,3-Gal epitope compared with naive mice, this subpopulation of B cells was unchanged in $HT^{AT20}$-grafted mice.

## CONCLUSIONS AND SIGNIFICANCE

Many human diseases and injuries result in tissue loss or dysfunction. The field of tissue engineering has progressed substantially in recent years to provide a therapeutic solution. Tissue-engineered cartilage is actively being pursued due to the limitations of available treatments to repair cartilage defects. The use of xenogeneic porcine cells or tissues for tissue engineering may lead to further advances and broaden its clinical application, but immunological hurdles need to be identified and addressed appropriately for each type of tissue. To gain insight into the mechanism of xenogeneic cartilage rejection, we first studied the contribution of $CD4^+$ T cells in wild-type mice grafted with porcine cartilage. $CD4^+$-T cell depletion demonstrated that the elicited anti-pig antibody response is T cell dependent in wild-type mice. The grafts showed a dramatic reduction in the amount of cellular immune infiltrate, particularly monocytes/macrophages. These observations agree with previous reports identifying a critical role of $CD4^+$ T cells in rejection of other xenogeneic tissues. Rejection of xenografts is known to be predominantly a Th2 response, and our results indicate that a T cell-mediated humoral response plays a role in rejecting porcine cartilage. We believe that several mechanisms that lead to delayed xenograft rejection in solid organs are common to the rejection

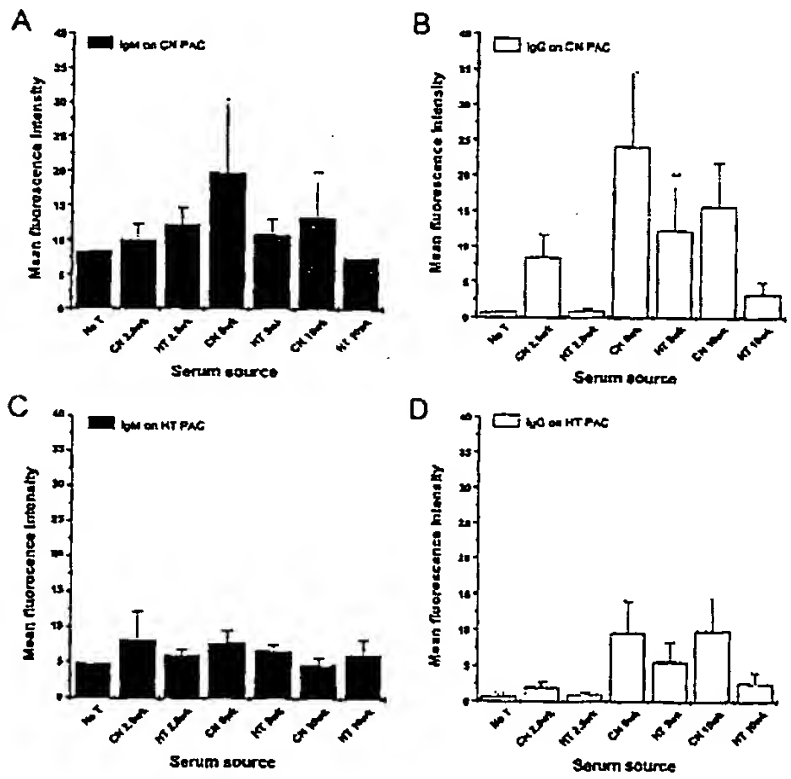


Figure 2. Flow cytometric analysis of anti-PAC antibody reactivity in sera from Gal KO mice transplanted with control (CN) or $HT^{AT20}$ transgenic grafts (HT). IgM (*A, C*) and IgG (*B, D*) reactivity of 10% heat-inactivated sera to control (*A, B*) and HT PAC (*C, D*) is shown as mean of mean FL-1 fluorescence intensity. Results correspond to 5 nontransplanted mice (No T), 2 mice grafted with control or HT grafts for 2.5 wk, 7 mice grafted with control or HT grafts for 5 wk, and 6 mice grafted with control or HT grafts for 10 wk. Error bars indicate the standard error of the mean.

process of xenogeneic cells and tissues. The Galα1,3-Gal antigen is the major xenoantigen in porcine tissues recognized by human natural antibodies. The contribution of the Galα1,3-Gal antigen to delayed xenograft rejection and delayed tissue rejection is not well established. To address this question, we transplanted control or HT transgenic engineered cartilage into Gal KO mice. We demonstrated that control cartilage expresses Galα1,3-Gal antigen whereas its expression is dramatically diminished in HT transgenic cartilage. Control porcine cartilage was rejected in several weeks by a prominent cellular immune infiltrate and elevated anti-pig and anti-Galα1,3-Gal antibody titers. In contrast, Gal KO mice receiving the HT cartilage showed a markedly reduced immune response, consistent with a major role of Galα1,3-Gal antigen in mediating delayed rejection of tissues.

To understand the Galα1,3-Gal-mediated tissue rejection process, we need to keep in mind how the anti-Galα1,3-Gal antibody response is generated in Gal KO mice. The anti-Galα1,3-Gal IgM response is independent of T cell help, but Galα1,3-Gal binding B cells that recognize glycoproteins containing this antigen need to bind to T cells for further activation and isotype switching. We have observed an increase in the quantity of Galα1,3-Gal binding B cells in spleen of control grafted mice, but not in Gal KO mice receiving HT grafts. An increase in this population of B cells has been described in Gal KO mice immunized with Galα1,3-Gal-containing antigens. Moreover, these cells are major producers of anti-Galα1,3-Gal IgM antibodies. As there was no anti-Galα1,3-Gal antibody response of either IgM or IgG subtypes in the HT-grafted Gal KO mice, the response was most likely absent due to insufficient Galα1,3-Gal-mediated B cell activation. Furthermore, the anti-pig antibody response was markedly reduced in this cohort. Apart from Galα1,3-Gal, no other carbohydrates seemed to play a major role in the elicited IgM response in this animal model. The lower anti-pig IgG antibody response of the HT-grafted Gal KO mice could be explained by a decreased presentation of xenoantigens to T cells. Our results imply that the Galα1,3-Gal binding B cells play a role as antigen-presenting cells that amplify the T cell response. In summary, different pathways may have contributed to the survival of the HT grafts in the Gal KO mice. First is a reduction in natural antibody reactivity, as we determined a decrease in Gal KO-mouse XNA deposition on HT PAC relative to control cells. Second, the anti-Galα1,3-Gal antibody response was eliminated and the anti-pig antibody response was reduced. A concomitant decrease in complement activation and antibody-dependent cell-mediated cytotoxicity would also be expected. Other mechanisms such as reduced cell interactions to HT grafts by NK cells and macrophages due to specific carbohydrate recognition may have played a role, but those direct cellular reactions remain to be elucidated in Gal KO mice.

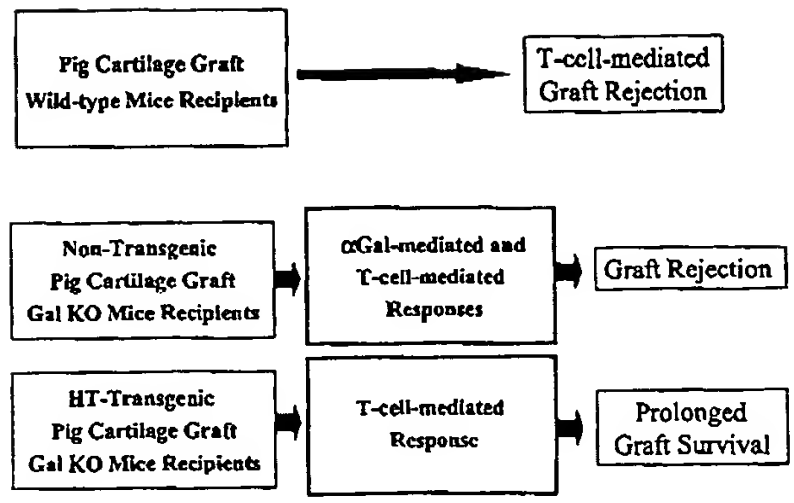


Figure 3. Schematic diagram summarizing the process of xenogeneic porcine cartilage rejection. The dramatic effect of depleting $CD4^+$ T cells on porcine graft survival in wild-type mice suggests this is mainly a T cell-mediated rejection process. In Gal KO mice receiving nontransgenic porcine cartilage, the rejection is amplified by a Galα1,3-Gal (αGal) -mediated response. The marked reduction in Galα1,3-Gal antigen expression in HT transgenic cartilage grafts is sufficient to abrogate Galα1,3-Gal-mediated immune rejection in Gal KO mice.

Our observations have clear relevance to the pig-to-human clinical setting, although there are probably differences to be considered. In some cases, the human system has been better characterized than the mouse. We have observed that transgenic expression of HT decreases human NK cell-mediated cytolysis of fibroblasts and monocyte cell adhesion to PAC. To evaluate these effects, this strategy should ultimately be tested in a primate model. Nevertheless, our results are consistent with those of Stone et al., where porcine cartilage was treated with α-galactosidase to remove the Galα1,3-Gal antigen and transplanted into Cynomolgus monkeys. However, they observed no reduction in the anti-pig antibody titers relative to control-cartilage recipients. Our approach is advantageous because it does not involve treatment of the tissue and prevents the cells from producing more Galα1,3-Gal antigen. It is also preferable to other strategies based on modifying the recipient. We believe that expression of HT by genetic engineering of the animal donor has real potential for contributing to xenograft acceptance in cell- and tissue-based clinical applications. Numerous applications may benefit from combining the progress in the fields of tissue engineering and xenotransplantation. Porcine cartilage either as cartilage plugs, in vitro engineered with/without scaffolds, or isolated chondrocytes injected with hydrogels could be used for orthopedic and reconstructive surgery. Here we show that reduction of the Galα1,3-Gal antigen on cartilage tissue by high expression of HT inhibits the elicited antibody response and delays rejection. Additional genetic modifications of the donor tissues designed to inhibit the cellular immune responses may eventually eliminate the need of immunosuppression. FJ

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TRANSPLANTATION

Vol. 65, 832–837, No. 6, March 27, 1998
*Printed in U.S.A.*

# TRANSGENIC EXPRESSION OF HUMAN α1,2-FUCOSYLTRANSFERASE (H-TRANSFERASE) PROLONGS MOUSE HEART SURVIVAL IN AN EX VIVO MODEL OF XENOGRAFT REJECTION

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***Background.*** **The expression of human α1,2-fucosyltransferase (H-transferase, HT) has been proposed as an alternative strategy to α1,3-galactosyltransferase (GT) gene knockout, which is not currently feasible in pigs, to reduce the galactose-α1,3-galactose (Gal) epitope expression. HT expression has recently been shown in transgenic mice and pigs to significantly reduce Gal expression on a variety of cells; however, its ability to do so on endothelial cells and its effectiveness at prolonging xenograft survival are yet to be determined.**

***Methods.*** **HT-transgenic, Gal knockout (Gal KO) mice, and mice containing both genetic modifications (HT-transgenic/Gal KO) were tested for H-substance and Gal expression on splenocytes and endothelial cells by flow cytometric analysis. In addition, the hearts of these mice were perfused ex vivo with 6% human plasma, and the effect on cardiac function was determined.**

***Results and Conclusion.*** **H-substance expression was detected on both splenocytes and endothelial cells of HT-transgenic mice. The level of H-substance expression was not affected by the presence or absence of GT in the cells, consistent with HT being dominant over GT. The ability of HT expression to reduce Gal expression was highly variable depending on the cell type. Gal expression on splenocytes was almost completely eliminated, whereas on endothelial cells, substantial Gal remained despite a 70% reduction. When perfused ex vivo with human plasma, hearts from HT-transgenic, Gal KO, and HT-transgenic/Gal KO mice demonstrated a similar prolongation in survival, compared with wild-type controls. Therefore, as far as hyperacute rejection is concerned, HT expression may be as effective as Gal KO in protecting against xenoantibody and complement mediated injury. However, the effect of residual Gal on non-hyperacute rejection responses remains to be determined.**

The transplantation of pig organs to humans is currently regarded as a possible solution to the chronic shortage of human donor organs available for transplantation. However, at present xenografts invariably fail due to hyperacute rejection (HAR*) (*1*, *2*). It is now clear from a variety of in vitro and in vivo studies that the binding of naturally occurring xenoantibodies to the disaccharide, galactose-α1,3-galactose (Gal) on vascular endothelium of the xenograft is a key mediator of xenograft rejection and, therefore, that elimination of this interaction will be highly beneficial to overcoming HAR (*3–8*). The strategies that have been proposed to achieve this goal can be broadly classified as recipient or donor directed (reviewed in *9*). The disadvantages of the former are that they are temporary and, in general, rely on transient depletion to achieve long-term xenograft survival. Furthermore, they impose an additional "therapeutic burden" on the recipient. These limitations have prompted the development of genetic-based strategies to eliminate or reduce Gal expression on the donor organs. We have previously reported the generation of mice in which the α1,3-galactosyltransferase (GT) gene has been inactivated by homologous recombination (Gal KO), resulting in complete elimination of Gal expression (*10*). Hearts from these mice demonstrated a significant prolongation in survival compared with wild-type controls when perfused ex vivo with human plasma (*11*). However, it is currently not possible to generate Gal KO pigs because porcine ES cells, which are critical for this technology, are not yet available. Consequently, enzymatic remodeling using readily available pig transgenic technologies has been proposed as an alternative strategy to modulate the expression of the Gal epitope (*12*, *13*). Transgenic mice and pigs expressing human α1,2-fucosyltransferase (HT), an enzyme which has the same substrate specificity (*N*-acetyllactosamine) as GT, have been generated by a number of groups and demonstrated significantly reduced Gal expression (*14–17*). However, none of the studies performed to date have examined the ability of HT to suppress Gal expression on endothelial cells or the effectiveness of this strategy at prolonging xenograft survival. In this study, cardiac endothelial cells were isolated from HT-transgenic mice, and the effect on Gal epitope expression was examined. In addition, the functional effect of HT expression was studied using an ex vivo heart perfusion model and compared with complete elimination of Gal by gene inactivation (Gal KO).

## MATERIALS AND METHODS

*Generation of experimental animals.* The generation of HT transgenic mice, in which human HT cDNA is expressed under the control of the murine $H2K^b$ promoter, has been previously described (*15*).

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* Abbreviations: FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; Gal, galactose-α1,3-galactose; GS-$IB_4$, *Griffonia simplicifolia* I-$B_4$ lectin; GT, α1,3-galactosyltransferase; HAR, hyperacute rejection; HT, H-transferase; KO, knockout; MCF, mean channel fluorescence; UEA, *Ulex Europaeus agglutinin* lectin; WT, wild type.

The generation of $\alpha$1,3 galactosyltransferase Gal KO mice by homologous recombination has also been described (*10*). The two mouse lines were crossed, and $HT^{=}/GT^{=}$ progeny were backcrossed with either wild-type or Gal KO mice to generate four test groups of mice with genotypes: $HT^{-/-}/GT^{+/+}$ (WT), $HT^{-/+}/GT^{+/+}$ (HT-transgenic), $HT^{-/-}/GT^{-/-}$ (Gal KO), and $HT^{-/+}/GT^{-/-}$ (HT-transgenic/Gal KO), respectively. The genotypes of the mice were determined by PCR and flow cytometric (fluorescence-activated cell sorter, FACS) analysis as described previously (*10*, *15*). All of the HT-transgenic mice used were hemizygous for the HT transgene.

*FACS analysis of splenocytes.* Single cell splenocyte suspensions obtained by sieving were incubated in 0.9% $NH_4Cl$ solution at 42°C for 5 min to lyse red blood cells and centrifuged. Cells were fixed in 4% paraformaldehyde on ice for 5 min, washed twice in Hanks' balanced salt solution (Sigma, St. Louis, MO), and then incubated with fluorescein isothiocyanate (FITC) conjugated *Griffonia simplicifolia* I-$B_4$ lectin (GS-$IB_4$) (20 $\mu$g/ml, Sigma) or *Ulex Europaeus agglutinin* lectin (UEA) (20 $\mu$g/ml, Sigma) on ice for 30 min. Cells were washed twice with phosphate-buffered saline and analyzed with a FACSCalibur (Becton Dickinson, Sunnyvale, CA).

*Preparation and FACS analysis of cardiac endothelial cells.* Hearts were excised, dissected into small pieces, and then incubated in a solution containing Dispase II (1.8 U/ml, Boehringer Mannheim GmbH, Mannheim, Germany), DNase (Boehringer Mannheim, 0.5 mg/ml), and 1% heat-inactivated fetal calf serum at 37°C for 2 hr. The tissue fragments were then digested at 4°C overnight with 2 mg/ml of collagenase B (Boehringer Mannheim) in Dulbecco's modified Eagle's medium/20% heat-inactivated fetal calf serum. The resultant cell suspension was washed twice in Hanks' balanced salt solution and incubated sequentially with rat anti-mouse PECAM-1 antibody (a gift from Dr. R. Boyd, Monash University, Melbourne, Australia), biotinylated goat anti-rat IgG (1/100; Rockland Gilbertsville, PA), and finally Streptavidin Tricolor (1/200; Caltag Laboratories, San Francisco, CA) and FITC-labeled GS-$IB_4$ or UEA lectins. Controls included unstained cells and cells stained with Tricolor in the absence of primary reagent. Endothelial cells were gated using fluorescence 3 for PECAM-1 expression and analyzed for Gal and H-substance expression using fluorescence 1.

*Preparation of human plasma and perfusion buffer.* Human plasma was prepared from normal blood donors, aliquoted, and stored at −70°C. On the day of the perfusion experiment, aliquots of plasma from three separate donors were thawed slowly, pooled, filtered through a 0.22-$\mu$m filter (Millipore Corp., Milford, MA), and stored on ice until required. All plasma preparations were routinely tested for classical complement pathway activity by standard methods (*18*). The perfusion buffer (modified Krebs-Henseleit) consisted of 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 11 mM glucose, 1.2 mM $KH_2PO_4$, and 0.25% (w/v) bovine serum albumin at pH 7.4. The perfusion buffer was prepared fresh each day and filtered (0.22 $\mu$M) prior to use. Throughout the experiments, the buffer was maintained at 37°C and gassed continuously with a mixture of 95% $O_2$/5% $CO_2$.

*Ex vivo mouse heart perfusions.* Perfusion of mouse hearts with human plasma was performed essentially as described by Romanella et al. (*18*). Briefly, mice were anesthetized with pentobarbitone sodium, and the heart was surgically removed and immersed in 20 ml of ice-cold perfusion buffer containing heparin (140 IU, Sigma) to arrest cardiac activity. Hearts were then attached to a 19-gauge cannula via the aortic root, connected to the perfusion apparatus, and perfused in a retrograde manner at a pressure of 80–100 mmHg. Hearts were perfused with perfusion buffer alone for 20 min to stabilize cardiac function, and human plasma was then added to the perfusate pool at 5-min intervals to achieve final concentrations of 2, 4, and 6%, respectively. Heart rate and force of contraction were recorded using MacLab (AD Instruments, Castle Hill, Australia) and used to calculate an index of cardiac work, which was expressed as a percentage of cardiac work at $t=0$, defined as the time of first plasma addition.

*Immunohistology.* Hearts perfused with 6% human plasma for 20 min were removed from the perfusion rig, embedded in OCT, and snap-frozen in liquid nitrogen. Sections (4 $\mu$M) were cut and air dried onto gelatin-coated slides. The slides were preincubated with Tris-buffered saline containing 10% normal sheep serum for 60 min before addition of FITC-conjugated rabbit anti-human IgG (1:1000), IgM (1:100), or C3c (1:1000) and incubation at room temperature for 60 min (all antibodies were purchased from DAKO, Carpinteria, CA). The slides were washed and incubated with peroxidase-conjugated anti-FITC Fab fragments (Boehringer Mannheim. 1:300 dilution) for 30 min. Peroxidase staining was detected using 3,3'-diaminobenzidine tetrahydrochloride (Sigma), as the chromogen and sections were counterstained with Harris's haematoxylin. The slides were examined under a light microscope.

## RESULTS

*Expression of H-substance on splenocytes and endothelial cells of HT-transgenic mice.* Splenocytes and endothelial cells prepared from HT-transgenic and WT mice were analyzed for the expression of H-substance using UEA lectin, which is specific for terminal $\alpha$-L-Fuc residues. As shown in Figure 1, splenocytes and endothelial cells from the HT-transgenic mice were positive for UEA, with mean channel fluorescence (MCF) values of 44 and 224, respectively. However, a direct comparison of MCF levels between endothelial cells and splenocytes is not possible due to the major difference in cell size between these two populations.

*Effect of HT expression on Gal epitope levels on splenocytes and endothelial cells.* The effect of H-transferase expression on Gal-epitope expression on splenocytes and endothelial cells was examined by staining with GS-$IB_4$ lectin, which is specific for the $\alpha$-Gal residue. As shown in Figure 2, splenocytes and endothelial cells from WT mice were positive for GS-$IB_4$ with MCF values of 20 and 302, respectively. As expected, splenocytes and endothelial cells from Gal KO mice, which were included as Gal-epitope negative controls, reacted very weakly with GS-$IB_4$, with MCF values of 1 and 6, respectively (which represent nonspecific background staining). With splenocytes from HT-transgenic mice, GS-$IB_4$ lectin binding was reduced by greater than 90% to a level (MCF: 2) approximating that observed on splenocytes from Gal KO mice. HT expression also resulted in a significant reduction (70%) in GS-$IB_4$ binding to endothelial cells. However, in contrast to splenocytes, endothelial cells from HT-transgenic mice still bound significant levels of GS-$IB_4$ lectin

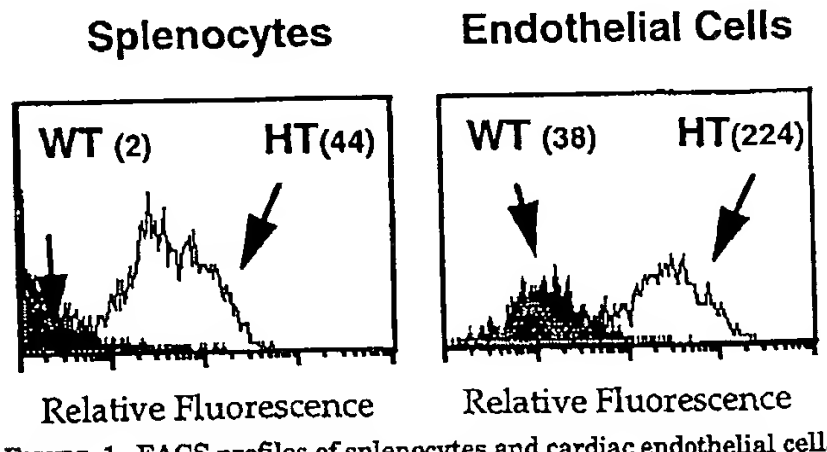
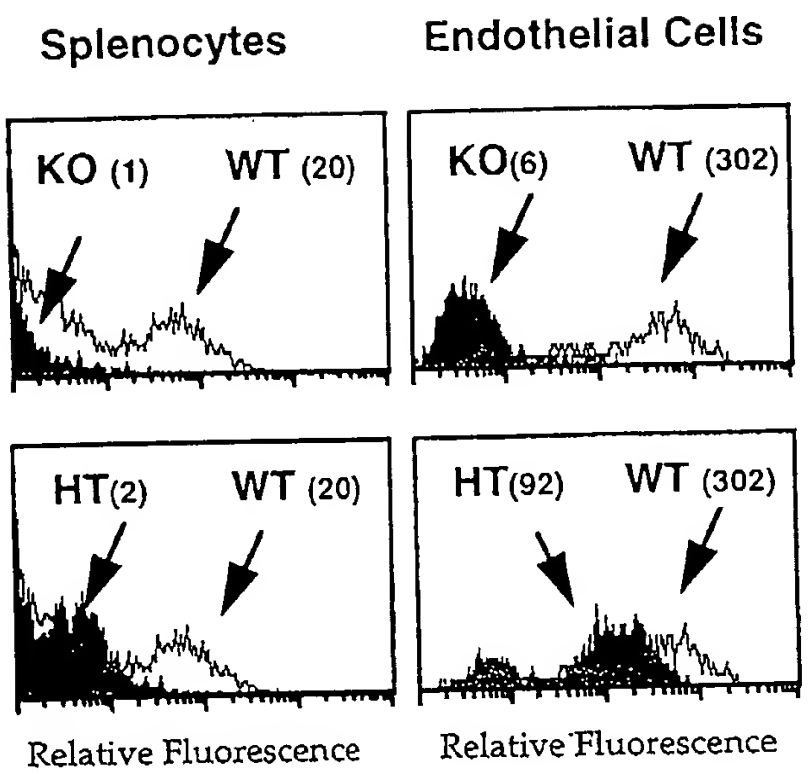



FIGURE 1. FACS profiles of splenocytes and cardiac endothelial cells from HT and WT mice stained with FITC-UEA. The mean channel fluorescence values are in parentheses. Results presented are representative of three separate experiments.



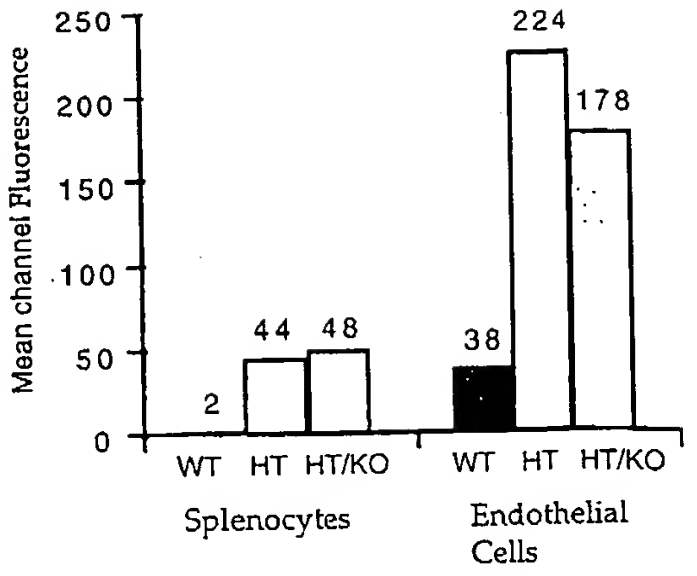




FIGURE 3. Expression of the H-substance on splenocytes and cardiac endothelial cells isolated from HT, HT/KO, and WT mice. Cells were stained with FITC-UEA and analyzed with a FACSCalibur. Results presented are representative of three separate experiments.

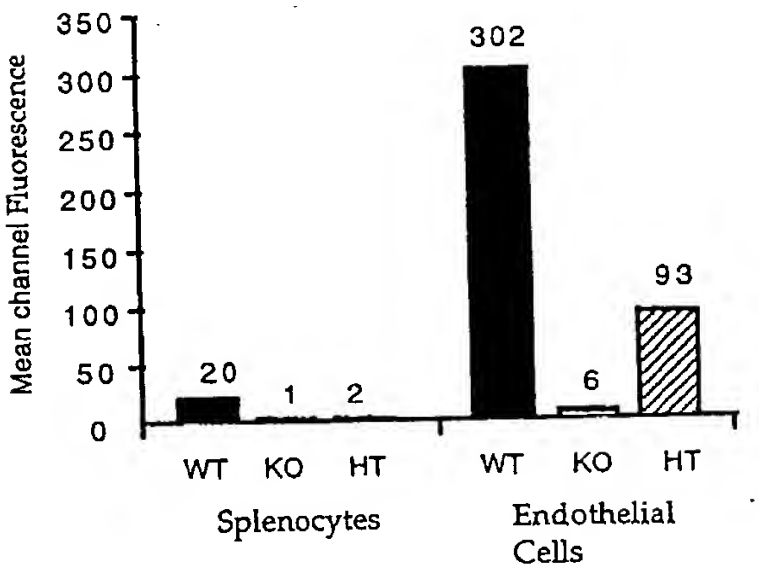



FIGURE 2. (Top) FACS profiles of splenocytes and cardiac endothelial cells from HT, KO, and WT mice stained with FITC-GS-IB$_4$. The mean channel fluorescence values are in parentheses. (Bottom) Graphic representation of FITC-GS-IB$_4$ mean channel fluorescence values. Results presented are representative of three separate experiments.

(MCF: 92), which is 10-fold greater than the level of binding to endothelial cells from Gal KO mice.

*Combination of Gal KO and HT expression.* To investigate the mechanism by which HT expression reduces Gal epitope expression, the HT-transgenic mice were crossed with Gal KO mice ($Gal^{-/-}$). Progeny which were heterozygous for both the HT transgene and the Gal KO allele ($HT^{-/+}/GT^{-/+}$) were identified by FACS and PCR analysis. These mice were then crossed with Gal KO mice, and progeny hemizygous for the Gal KO allele ($HT^{-/+}/GT^{-/-}$, HT-transgenic/Gal KO) were identified. As expected, splenocytes and endothelial cells from the HT-transgenic/Gal KO mice were negative for GS-IB$_4$, indicating complete elimination of the Gal epitope (data not shown). FACS analysis using UEA lectin showed that the level of H-substance expression on splenocytes and endothelial cells from HT-transgenic/Gal KO mice was similar to that detected on the same cells from HT-transgenic mice with a wild-type GT background (Fig. 3).

*Ex vivo heart perfusion.* Hearts from HT-transgenic, Gal KO, HT-transgenic/Gal KO, and WT mice were perfused with human plasma (6%) on a modified Langendorff perfusion apparatus. The cardiac work of hearts from WT mice (n=6)

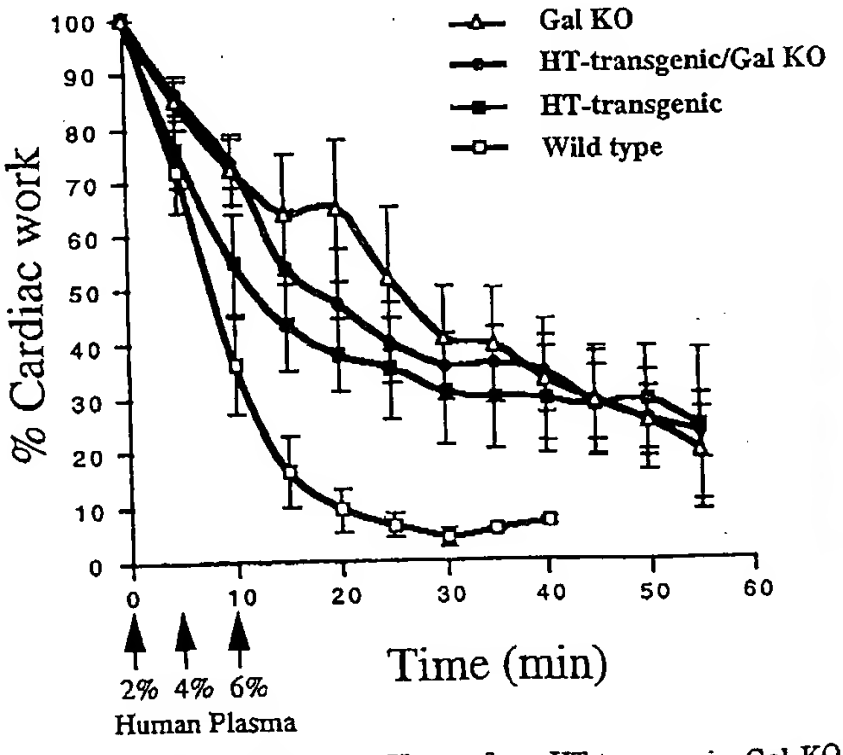



FIGURE 4. Ex vivo perfusion. Hearts from HT-transgenic, Gal KO, HT-transgenic/Gal KO and wild-type mice were perfused with human plasma. Work performed by the hearts was calculated as the product of the rate and force of contraction. Data are expressed as the percentage of work at the point shown compared with maximal work at $t$=0 (defined as the time of the first plasma addition). Results presented as mean ± SEM, n=6 in each group. The time points at which human plasma was added are indicated (arrowhead).

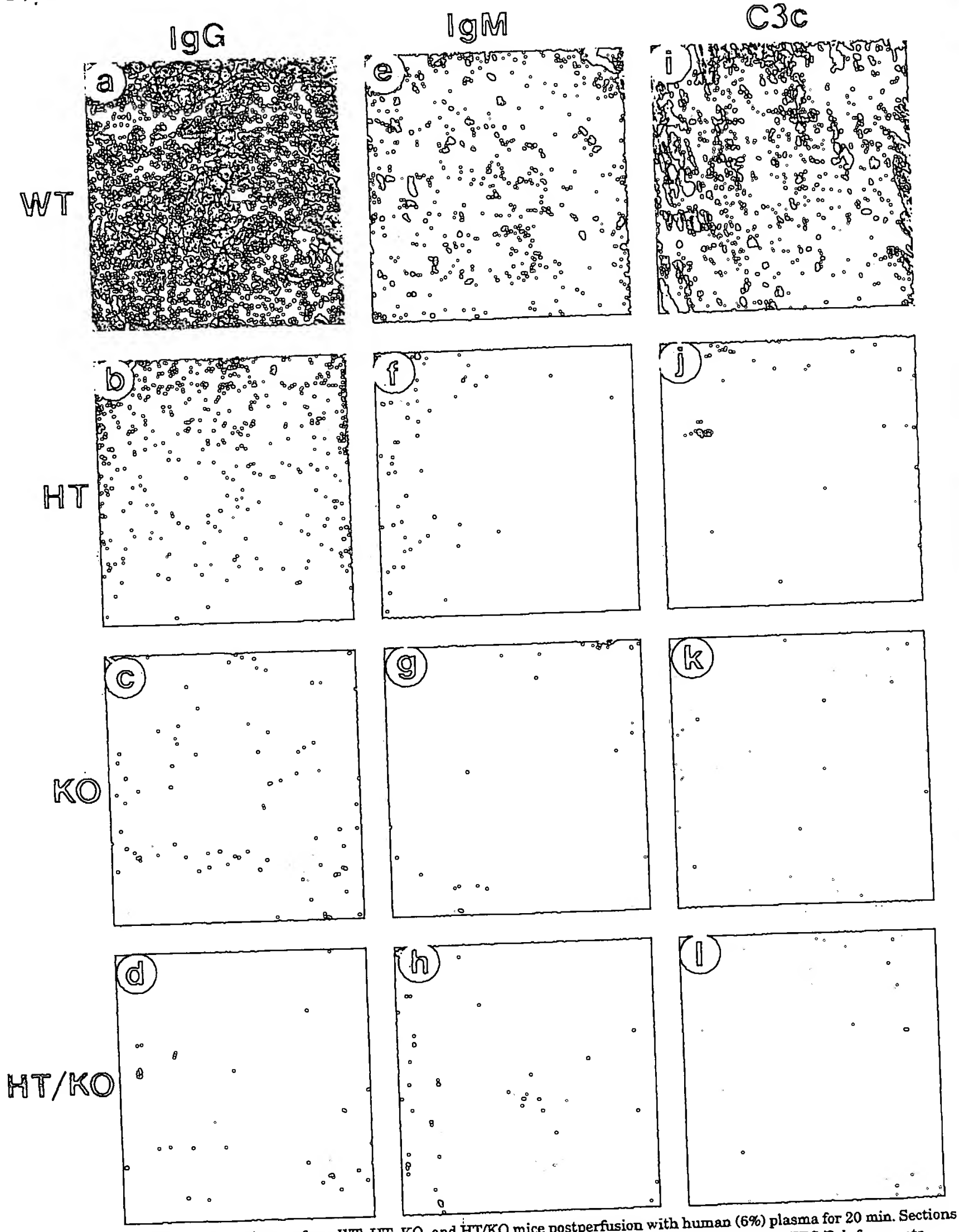



FIGURE 5. Immunohistochemistry of hearts from WT, HT, KO, and HT/KO mice postperfusion with human (6%) plasma for 20 min. Sections were stained with FITC-conjugated rabbit anti-human IgG, IgM, or C3c, followed by peroxidase-conjugated anti-FITC Fab fragments.

dropped sharply to below 10% of maximum heart work within 20 min of plasma addition. In contrast, hearts from HT-transgenic, HT-transgenic/Gal KO, and Gal KO mice (n=6 in each group) were still functioning at 40–60% of maximum after 20-min perfusion, with no significant difference between the three groups (Fig. 4). Furthermore, these hearts continued to function at approximately 30% of maximum after 55-min perfusion, at which point the experiment was terminated.

*Immunohistochemistry of perfused hearts.* Hearts perfused with 6% human plasma for 20 min were removed from the perfusion rig and examined immunohistologically for the deposition of human Ig and the activated complement component C3c (Fig. 5). Hearts from WT mice demonstrated strong staining for human IgG, IgM, and C3c (Fig. 5, a, e, and i). In Gal KO and HT-transgenic/Gal KO hearts, there was a marked reduction in IgG, IgM, and C3c deposition, in particular on the endothelium of large blood vessels (Fig. 5, c–l). A similar level of reduction in IgM and C3 deposition was also observed in HT-transgenic hearts (Fig. 5, f and j); however, in contrast to both groups of Gal KO mice, there was little or no reduction in human IgG deposition (Fig. 5b). As expected, no staining for IgG, IgM, or C3c was observed in hearts perfused with modified Krebs-Henseleit buffer alone, which were included as a negative control (data not shown).

## DISCUSSION

Transgenic expression of human H-transferase is currently viewed as the most feasible strategy to reduce Gal epitope expression in pigs. This strategy has been shown to significantly reduce Gal expression on a variety of cells in transgenic mice and pigs (*14–17*). However, its ability to do so on endothelial cells and its effectiveness at prolonging xenograft survival are yet to be demonstrated

In this study, we have shown that the capacity of H-transferase to reduce Gal epitope expression is cell type dependent. With splenocytes, Gal expression was reduced by greater than 90% to approximately background levels. In contrast, Gal expression on cardiac endothelial cells was only reduced by 70%. Consistent with the histological observations, isolated cardiac endothelial cells from HT-transgenic mice still demonstrated considerable GS-$IB_4$ reactivity. The explanation for the partial reduction on endothelial cells is not obvious. One possibility is that $\alpha$1,3-galactosyltransferase enzyme activity is higher in endothelial cells than in splenocytes. Alternatively, the relative ordering of $\alpha$1,3-galactosyltransferase and H-transferase in the Golgi may be different in endothelial cells compared with other cells (*19*), such that H-transferase is not dominant over $\alpha$1,3-galactosyltransferase. However, these explanations seem to be unlikely because the level of H-substance expression on both splenocytes and endothelial cells was the same in both HT-transgenic and HT-transgenic/Gal KO mice. Another possible explanation is that compared with splenocytes, endothelial cells contain more substrate (*N*-acetyllactosamine), which cannot be fully utilized by H-transferase at the level expressed in the transgenic line studied. This being the case, the obvious solution would be to use promoters that would give a higher level of HT expression. However, to date we have been unable to generate homozygous mice from this line of HT-transgenic mice or produce transgenic mice expressing high levels of H-transferase using the strong endothelial cell-specific intercellular adhesion molecule-2 (*20*) promoter (data not shown), suggesting that there may be "toxicity" problems associated with high levels of H-transferase expression. This may be related to the marked distortion in cell surface carbohydrate expression that has been observed on cells from HT-transgenic mice, including a significant reduction in $\alpha$2,3-sialylation (*21*).

The protective effects of reducing Gal epitope level by H-transferase expression were examined using a Langendorff ex vivo perfusion model. As expected, the cardiac function of hearts from wild-type mice perfused with human plasma (6%) dropped sharply to below 10% of original activity within 20 min of plasma addition. In contrast, the function of HT-transgenic, Gal KO, and HT-transgenic/Gal KO hearts was only reduced by 40–60% after 20 min, and cardiac output was maintained at approximately 30% in all three groups after 55 min, the point at which the experiment was terminated. In comparison, the cardiac output of control wild-type hearts perfused with Krebs-Henseleit buffer alone dropped to about 60% of maximum within the same period of time (data not shown), which represents the "natural" deterioration in cardiac function resulting from the ex vivo perfusion procedure. Immunohistochemical examination of hearts postperfusion demonstrated a marked reduction in human IgM and C3c deposition on endothelium of hearts from all three experimental groups compared with WT controls. Therefore, as far as HAR is concerned, the results of the present study would suggest that the H-transferase expression is as effective as Gal KO in protecting against human xenoantibody and complement mediated injury. Whether this is also the case in pigs where greater than 90% of human xenoantibody reactivity is directed at the Gal epitope, compared with 50–60% in mice (*11*), remains to be determined. It also remains to be determined what contribution the residual Gal epitopes, which may induce high titre anti-Gal antibody responses in the recipient (*22*), and the exposed crypt antigens (*21*) will make to post-HAR rejection mechanisms such as antibody-dependent cellular cytotoxicity. These issues are currently being addressed using a cardiac transplant model in which hearts from HT-transgenic mice are transplanted into Gal KO mice containing anti-Gal antibody.

*Acknowledgments.* The authors thank Helen Barlow, Vikki Smyth, and Nicolle Bruengger for the maintenance of mice used in this study.

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Received 2 September 1997.
Accepted 19 November 1997.

0041-1337/98/6506-837$03.00/0
TRANSPLANTATION


Vol. 65, 837–843, No. 6, March 27, 1998
*Printed in U.S.A.*

# A CRUCIAL ROLE OF HOST CD80 AND CD86 IN RAT CARDIAC XENOGRAFT REJECTION IN MICE

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***Background.*** **Graft rejection can be initiated by two primary pathways of antigen presentation: (a) direct activation of host T cells by donor-derived antigen presenting cells (APC) and (b) indirect presentation of processed graft antigens by host APC.**

***Methods.*** **We investigated the differential roles for direct and indirect antigen presentation by preventing the CD28 costimulatory pathway with monoclonal antibodies to rat or mouse CD80 and CD86 in a rat-to-mouse cardiac transplantation model.**

***Results.*** **Although the mouse anti-rat monoclonal antibodies to CD80 and CD86 did not significantly prolong the survival of rat cardiac xenografts in mice, the rat anti-mouse monoclonal antibodies to CD80 and CD86 did prolong the survival. Development of the anti-donor antibodies was inhibited, and the deposition of C3, IgM, and IgG on endothelium in the xenografts was mild in the anti-mouse CD80/CD86-treated mice. Infiltration of macrophages, neutrophils, and lymphocytes expressing perforin and interferon-γ was decreased by the anti-mouse CD80/CD86 treatment.**

***Conclusions.*** **These findings suggest that the indirect antigen presentation, which is mediated by CD80 and CD86 pathway on host APC, plays a crucial role in concordant cardiac xenograft rejection.**

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The increasing problem of the shortage of donor organs has led to a revisal of interest in xenotransplantation (*1*). Xe-